PROCEEDINGS

OF THE

ROYAL SOCIETY OF LONDON

SERIES B—BIOLOGICAL SCIENCES

VOL CXVIII

LONDON
Printed and published for the Royal Society
By Harrison & Sons, Ltd., 44–47, St. Martin's Lane
Printers in Ordinary to His Majesty

November 1935

LONDON:
HARRISON AND SONS, LTD., PRINTERS IN ORDINARY TO HIS MAJESTY
ST. MARTIN'S LANE

CONTENTS

SERIES B VOL CXVIII

No. B 807—July 1, 1935

No. B 808—August 1, 1935

A Hormone causing Pupation in the Blowfly *Calliphora erythrocephala*

By Gottfried Fraenkel

From the Department of Zoology and Comparative Anatomy, University College, London

(*Communicated by D. M. S. Watson, F.R.S.—Received November* 18, 1934—*Revised March* 16, 1935)

[Plate 1]

Introduction

Excellent descriptions exist of the morphological and chemical changes in the metamorphosis of insects. But until the last few years practically nothing was known about the actual forces which induce, initiate, or control moulting and pupation. In growth and metamorphosis of vertebrates the dominant controlling role of hormones becomes more and more obvious. It is especially in changes which concern simultaneously the whole body that the action of hormones is involved. The metamorphosis of insects consists also of profound changes on the whole animal which manifest themselves even more abruptly than do analogous processes in vertebrates. But it was only about 10 years ago that the action of a hormone in the metamorphosis of insects was suggested for the first time by Kopeč (1922). The matter was raised again by von Buddenbrock (1930, 1931) and Koller (1929), a preliminary report having appeared in Koller's survey on hormones in invertebrates. Recently new evidence has been brought forward by Bytinski-Salz (1933) and Bodenstein (1933, *a* and *b*). The present position of the problem is given in an excellent review by Bodenstein (1933, *b*), and he comes to the conclusion that the action of hormones in insect metamorphosis can be shown to be very probable from several facts, even if definite proofs are still lacking. It may be mentioned here that both Koller and Bodenstein failed to recognize the important and convincing results of Kopeč.

A preliminary report of the present paper appeared in 'Nature' (Fraenkel, 1934).

Experiments and Observations

Preliminary Remarks and Technique Adopted

When reared at room temperature of about 20° C, the young larvae of *Calliphora erythrocephala* hatch from the egg about 24 hours after egg-laying, within a further 4 days they reach their maximal size and weight. In this state, which will be referred to as the mature larva,* the larva stops feeding, the crop becomes gradually emptied and reduced. This stage lasts about 3 days, then pupation starts. About 2 hours before pupation, the external appearance of the larvae becomes changed. Up to this time the skin looks shiny, it then becomes matt white. The movements gradually slacken and the shape becomes relatively shorter and broader. If the first individuals of a culture, all specimens being of the same age, are about to pupate, it can be expected with certainty that 70 to 80% of the whole batch will pupate within the following 24 hours.

The act of pupation itself is a very complicated process, consisting of several different reactions between which the connections are still entirely obscure. Roughly three reactions may be distinguished externally

(1) the well-known barrel shape of the pupa is assumed (white pupa);
(2) within a few hours the skin (cuticle) becomes hard;
(3) at the same time the skin darkens.

The two latter reactions - for some reason not yet known - seem to be indissolubly coupled. Usually the conditions of the experiment prevent the use of the former reaction (1) as a criterion for pupation. In the following statements, therefore, by pupation is understood the hardening and darkening of the skin. The complicated processes of histolysis and histogenesis which follow the actual pupation are not considered in the present paper.

The changes in the larval cuticle described above, whereby the puparium is being formed, have always been called pupation and will be referred to in this paper by this term. But it should be kept in mind that they represent really only the first inception of pupation. About 20 hours later the larval hypodermis becomes separated from the cuticle and during the second day after the formation of the puparium the real pupa is

* This stage has been called in my preliminary note (Fraenkel, 1934) prepupa in accordance to a definition given by Wardle (1930). But the very similar term pre-nymph has already been used by Lowne for the stage between the formation of the puparium and the formation of the pupa. The term resting larva, used by Lowne, does not adequately describe this stage, since the larvae are continuously moving about. For these reasons I adopted the term mature larva, used by Hewitt (1914).

formed by evagination and prolongation of the cephalic and thoracic imaginal discs.

One of the chief methods used was a functional separation of prepupae into two or three parts by tight ligatures of cotton or silk thread.

In the course of the work the necessity arose to inject blood of mature larvae into others. This was attended at first by great difficulties, since on piercing the cuticle a part of the body content flows out. This difficulty was finally overcome by the following arrangement: the front part of a larva was ligatured off lightly with only one knot and cut off in front of the knot. Then the blood of another larva was pressed out on a glass plate and sucked into a small glass pipette. The pipette was introduced into the larva through the knot and the fluid driven in. As the pipette was removed, the knot was tightened.

Ligaturing Experiments

If old mature larvae of *Calliphora erythrocephala* are ligatured into two parts, four different results may arise: (1) both the anterior and posterior part pupate. This occurs only within about 16 hours after the ligature is made (at 20° C). The front part is usually but not always in advance of the hind part by 1–2 hours, fig. 1, Plate 1. (2) Only the hind part pupates within about 16 hours. This case occurred comparatively seldom (only 11 times out of many hundred larvae ligatured), fig. 2, Plate 1. (3) Only the front part pupates, the hind part remaining unpupated. This result occurs only after more than 16 hours from ligaturing, fig. 3, Plate 1. (4) Neither the anterior nor the posterior part pupates.

These facts are illustrated by a review of a great number of experiments, Table I.

Table I

Time of pupation after ligaturing	Number of specimens		
	Both parts pupate	Anterior part alone pupates	Posterior part alone pupates
hours			
1–10	176	—	11
10–16	34	2	—
16–20	12	40	—
20–24	1 ?	30	—
24–36	—	71	—
36–48	—	81	—
48–72	—	82	—
72–96	—	8	—

Unfortunately the experiments could not be carried out at a consta temperature, but at room temperatures of 15–20° C. In all the 12 tes. when both parts pupated after 16–20 hours, the temperature was exceptionally low (10–15° C), so that in no case did pupation of the hind part occur after more than 16 hours at 20° C.

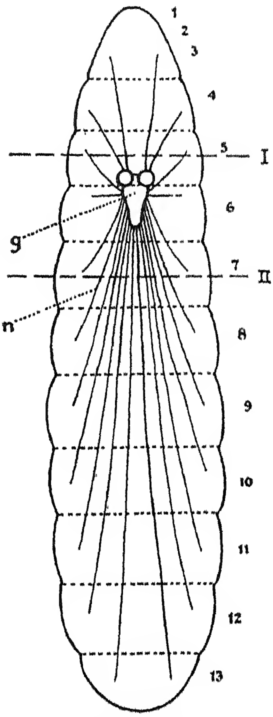


FIG. 4—Nervous system of the larva (after Hewitt). *g*, ganglionic mass; *n*, nerves; 1–13, body segments; I, ligature in front of the ganglion; II, ligature behind the ganglion

The results of these experiments are the same whatever be the position of the ligature, provided that this be placed behind the sixth segment in the sense stated by Hewitt (1914). In actual practice this means behind the fourth of the grooves which surround the larva. If this condition be met the anterior part always contains all the ganglia which are concentrated in the fly larvae into a single mass roughly between the fifth and sixth segment, fig. 4. For convenience this ganglionic mass will be referred to as the "ganglion," a term introduced by Hew (1914).

But if the ligature is laid down in front of the fifth segment (*i.e.*, the "ganglion"), the hind part behaves exactly like the front part in the experiments described above, both parts pupating within about 16 hours after ligaturing or the hind part alone pupating more than 16 hours after ligaturing, figs. 5 and 6, Plate 1. The presence of the "ganglion" can easily be recognized by the shape of the pupated parts. Only the part which contains the "ganglion" becomes properly barrel-shaped, whereas with the "ganglion" missing pupation consists merely of hardening and darkening of the skin, without change of the shape (owing to the complete lack of nervous centres in the latter case).

It seems superfluous to add that the results are exactly the same if one of the parts is entirely cut off after ligaturing.

In both the ligatured anterior and posterior parts, the development goes on after the formation of the puparium. The hypodermis becomes separated from the cuticle. In the front part by evagination of the imaginal discs the head, thorax, and appendages of the pupa are formed, in the hind part the larval tracheal trunks are withdrawn from the abdomen. Therefore the use of the term pupation does not merely

than formation of the puparium, but it implies formation of the pupa. These conditions are still under investigation.

From these experiments the following conclusions can be drawn.

There is something in the anterior part which induces or initiates pupation in the posterior part. About 16 hours before pupation this induction is already accomplished. Therefore a part separated from the source of the induction within this period is able to pupate. But if the ligature is made more than 16 hours before pupation, inducement to pupate cannot reach the hind part. The front part pupates if it lives long enough; this may happen even 3 days after ligaturing. But usually the anterior part, which failed to pupate, perishes after a few days, whereas the posterior part remains alive for a much longer time. In the few cases where only the posterior part pupated the ligature was laid down less than 10 hours before pupation. But an unknown factor inhibited the pupation of the front part. A suggested explanation of this peculiar behaviour is given on p. 9.

It follows with sufficient clarity from these experiments that the inducement to pupate originates either in the " ganglion " itself or in its immediate neighbourhood. The presence or absence of the " ganglionic " region determines the fate of the ligatured part.

Since the ligatured hind part contains only nerves and no ganglia, then the presence of the " ganglion " is not required for pupation unless it was removed more than 16 hours before.

Evidence for the Action of a Hormone Initiating Pupation

There are two possibilities concerning the nature of the stimulus to pupate. It may consist of a nervous stimulus brought to the skin by the nervous system, or of a special hormone, secreted in the front part and carried about by the blood. In order to decide this question, the following experiments were carried out.

(1) Old mature larvae were ligatured tightly and the ligature was removed after about 1 hour. In these specimens the nervous conduction between the two parts of the body is interrupted, but the blood circulates freely between them. The interruption of the nervous conduction can be recognized easily by the external appearance of the larvae. Only the part in front of the ligature continues to move about, whereas the part behind it becomes, and remains, motionless owing to the lack of nerve centres. At the same time controls of the same age were ligatured and the ligature left until pupation. If we suppose that the induction of

pupation consists of a nervous stimulus, then in all the experiments where pupation takes place more than 16 hours after ligaturing only the anterior part would pupate. The result would be the same in the specimens in which the ligature was removed as in those in which the ligature was left. But if the induction is carried out by a hormone, a different result would arise. The two parts of a larva would pupate simultaneously, if the blood circulates freely between them, even though their nervous connection is severed.

The result was unambiguous. In all specimens where nervous connection between the front and hind part was broken and the blood circulation restored, pupation occurred simultaneously in both parts of a larva. The front part never pupated alone. Altogether 34 specimens thus treated pupated more than 20 hours after ligaturing, 6 after 20–24 hours, 28 after 24–72 hours. In all these specimens the hind part would never have pupated if the ligature had been left. Of the controls, 29 pupated more than 20 hours after ligaturing and in all these only the anterior part pupated.

(2) In order to ascertain if the agents initiating pupation are carried about by the blood, injection experiments were undertaken. The technique is described on p. 3. After overcoming many difficulties, positive results were gained with two different arrangements. In all the experiments blood of larvae which were about to pupate within 2 hours was injected into posterior parts of younger ones.

(*a*) Hind parts of old mature larvae which would apparently have pupated after about 16 hours were injected with "pupation-blood." Twenty-four hours after injection those which had not yet pupated and would hence never have pupated were injected a second time. Of 44 twice-injected specimens 18, *i.e.*, 41%, pupated within 12 hours after the second injection. In these specimens only something contained in the injected blood can have induced pupation.

(*b*) Later the experiments were carried out with a simpler and more conclusive arrangement. Old mature larvae were ligatured and from 20 hours later those of them were picked out in which the anterior part alone had pupated. These posterior parts (which normally never would be able to pupate) were injected with "pupation-blood." The results of 12 different sets of experiments are summarized in Table II. The experiments were carried out partly at room temperature of about 20°, partly at 26° C. Group 1 consists of those in which the front part pupated within less than 28 hours, and Group 2 of those in which the front part pupated within 35–48 hours after ligaturing. One experiment in which the front parts alone pupated within 30–40 hours after ligaturing at room temperature is

included in Group 1, because at 26° C their pupation would have been expected at a much earlier point.

The result of the injections is very different in the two groups. In Group 1 of 48 injected hind parts, 22, *i.e.*, 46%, pupated, in Group 2 of 28 injected hind parts only 2, *i.e.*, 7%, pupated. This result indicates that pupation of the posterior parts of mature larvae can be induced by the injection of pupation blood where the stage of development of the larvae is such that pupation of the anterior half takes place within 24 hours. While if the larvae were ligatured at an earlier stage of development, such that 35–48 hours were necessary for the pupation of the anterior ends, injection of pupation blood usually failed to induce pupation of the posterior half. All pupations induced by injection occurred within less than 24 hours after injection.

TABLE II—INDUCTION OF PUPATION BY INJECTION

Time after ligaturing when front part alone pupated hours	Number of injected hind parts	Number of pupated hind parts	Number of unpupated hind parts	Number of deaths	Temperature
		GROUP 1			
Less than 24	10	4	3	3	Room
Less than 24	1	1	—	—	Room
Less than 24	2	1	—	1	Room
30–40	10	5	3	2	Room
Less than 28	9	1	5	3	26° C
Less than 28	11	7	2	2	26° C
Less than 24	5	3	—	2	26° C
	48	22			
		GROUP 2			
35–48	4	—	4	—	Room
35–48	5	—	?	?	Room
35–48	9	—	4	5	26° C
35–48	4	1	3	—	26° C
35–48	6	1	4	1	26° C
	28	2			

From these results two conclusions may be drawn—

(1) the pupation-inducing principle becomes ineffective after somewhat less than 24 hours;

(2) in ligatured larvae the anterior part develops further towards pupation, whereas the posterior part remains in the stage at which

it was ligatured. This follows from the fact that injection of pupation blood has different effects on ligatured posterior ends according to whether the front parts pupated 24 or 48 hours after ligaturing.

Since pupation can be induced by two subsequent injections, it would em possible to develop hind parts of young mature larvae into pupae y several consecutive injections. Unfortunately this experiment cannot e carried out owing to the high death rate of the injected animals.

The Site of Oxygen Uptake during Pupation

Since the discovery of tyrosinase in the blood of insects by Dewitz in 902, the colouring of the insect skin after moulting or metamorphosis is nderstood as a fermentative process. A chromogen, probably tyrosin, s converted by the action of the ferment tyrosinase into melanin in the resence of molecular oxygen. The necessity of oxygen is easily shown y placing white pupae in an atmosphere of pure N_2 or H_2 or CO_2. Here hey remain absolutely white for hours. Colouring starts immediately afterwards if oxygen is allowed to act upon them.

In the larval blood both chromogen and ferment are present. The blood very quickly becomes dark if exposed freely to the air. So far it is not clear how the colouring of the blood is prevented inside the body.

As to the mechanism of the colouring of the skin after moulting, it is generally believed that this process is provoked by the first exposure of the new—uncoloured—skin to the atmospheric oxygen. For the pupation of flies this view cannot be true, because the puparium is identical with the larval cuticle of the last moult. Therefore the colouring of the pupa can only be regarded as a consequence of changes inside the insect. If we pass over the nature of these changes, the question arises whether the oxygen required for the colouring process derives from the surrounding air, or from the air contained in the tracheal system.

For the solution of this question, it is a very favourable fact that in the fly larva only two parts of stigmata exist which are situated on the two ends of the body. If a white pupa is almost entirely immersed in oil, leaving only the posterior end with the hind spiracles in contact with air, the colouring and hardening occur simultaneously over the whole body at almost the same rate as normally. But if the reverse experiment is carried out, a different result occurs. At first only the part outside the oil pupates (*i.e.*, becomes dark and hard), then pupation spreads

gradually from the front end backward. During this process a colour gradient from the front to the hind end is visible, fig. 7, Plate 1. Finally the pupation reaches the hind end unless the animal dies before this. The colouring of the whole animal by means of the front stigmata requires about a day compared with a few hours in the normal case.

From these experiments two conclusions may be reached: (1) during pupation the oxygen necessary for the fermentative process is brought to the skin through the tracheal system; (2) the amount of air supplied through the posterior stigmata is greatly superior to that supplied through the front stigmata. This can easily be explained by the much larger size of the hind stigmata.

These conclusions are confirmed by the following experiment. When old larvae which are about to pupate are ligatured twice, into three parts, the anterior and posterior ends pupate successfully; but the middle part, the tracheal system of which is entirely cut off from the outside air, either does not pupate at all, or becomes only very slightly coloured, fig. 8, Plate 1.

We may find in these facts the key to the peculiar case in which only the posterior part pupated (p. 3). Possibly, the anterior part is prevented from pupation by lack of oxygen.

Discussion

The work up to the end of 1933 containing evidence for the presence of hormones causing metamorphosis in insects has been reviewed and discussed recently by Bodenstein (1933, *b*). It will be mentioned here only briefly so far as the present results are concerned. For the experiments of Buddenbrock (1930) the original paper must be consulted in which the results given are much less positive than in the preliminary report in Koller's review (1929). Buddenbrock injected blood of caterpillars which were due to moult or pupate within a short time into younger ones. These seemed to moult or pupate a little earlier than they would have done without injection. But a statistical review of the results showed that the difference was within the limits of experimental error. A much more positive result is contained in Bodenstein's experiments in which legs of caterpillars were transplanted into older specimens of the same instar. The implant always moulted simultaneously with the host. If legs of the last instar were transplanted into younger instars, an additional moult of the transplant was induced. These results show clearly that moulting is controlled by a substance contained in the blood.

The most important previous experiments to be mentioned here are those of Kopeć (1922). He deprived caterpillars of *Lymantria dispar* of the last instar of their supra-oesophageal ganglion. These caterpillars pupated if the ganglion was removed 10 days after the last moult or later, but failed to do so if the "brain" was removed 7 days after the last moult. The same conclusions resulted from ligaturing different parts of the body. "The front fragments undergo metamorphosis in a normal manner, provided the caterpillar does not die of starvation, but the middle and hind fragments undergo pupation only if they have belonged to caterpillars which would have pupated in a few days." When the nerve cord only was cut, the whole animal pupated simultaneously. Kopeć concluded from his experiments that a hormone is produced in the "brain" which induces pupation in the whole animal. This conclusion seems to require only a comparatively slight correction. It is not proved that the ganglion itself represents the hormone-producing organ. Two possibilities suggest themselves, either the secretion of a gland situated elsewhere may be induced by the "brain," or the gland may be represented in the corpora allata which are closely connected with the "brain."

This was the position of the problem about six months ago. All these researches were carried out with lepidopterous larvae.

Simultaneously with my researches on the blowfly the mechanism of moulting in the tropical bug *Rhodnius prolixus* was investigated by Wigglesworth (1934, *a*, *b*). Here definite proofs for the action of hormones causing moulting and metamorphosis have been established. There is a critical period, a certain time after feeding, before which moulting is prevented by decapitation, whereas bugs decapitated after this period do moult. "If the blood from an insect decapitated after this critical period is allowed to circulate in an insect decapitated before this period the latter is caused to moult." The source of the hormone is suggested to be the corpora allata, which undergo conspicuous changes during the critical period.

It seems, therefore, from the investigations of Kopeć, Wigglesworth, and the writer that the mechanism of moulting and pupation includes a very similar if not identical principle in three different insect orders, namely, in Lepidoptera (*Lymantria dispar*), Hemiptera (*Rhodnius prolixus*), and Diptera (*Calliphora erythrocephala*). At a definite period shortly before moulting or pupation a hormone is secreted in the anterior part of the body and carried about by the blood. Separated posterior parts only moult or pupate if the separation took place after this hormone was released and discharged. Disconnection of nervous transmission does

not prevent the hind part pupating if the blood circulation is maintained (*Lymantria* and *Calliphora*). Posterior parts separated before this period, which otherwise would not pupate, can be induced to pupate or moult by the injection of blood of larvae which have already passed this period (*Rhodnius*, *Calliphora*).

In *Lymantria* and *Rhodnius* the hormone is produced in the head, possibly in the corpora allata. In the headless fly-larva the source of the hormone was localized in the anterior part of the body in the immediate neighbourhood of the " ganglion." If we could assume that the " ganglion " itself produces the hormone, Kopeč's as well as my results would be satisfactorily solved. In the light of the new findings on " neurohumoralism " and secretory function in the brain of vertebrates, the metamorphosis of insects would form new, interesting evidence. But as long as proofs are lacking—it is stated above that Kopeč's results are not conclusive—the immediate neighbourhood of the " ganglion " in the fly-larva has to be searched for the hormone-producing organ. There exists no organ in the fly-larva which can be homologized with the corpora allata in other insects. The localization of the pupation-gland is the object of further investigation.

I take this opportunity of thanking the Academic Assistance Council for providing a grant, and Professor D. M. S. Watson for his continual interest and his hospitality in extending to me the facilities of his department.

Summary

Proofs have been established for the action of a hormone inducing pupation in the blowfly. This hormone is secreted from 16 hours before pupation, at 20° C. The hormone-producing organ is either the " ganglion " or in its immediate neighbourhood. After the hormone has already been discharged, pupation can be successfully accomplished without the co-operation of the nervous centres (ganglion).

The atmospheric oxygen required for the darkening of the pupa is brought to the skin through the tracheal system.

References

Bodenstein, D. (1933, *a*). 'Roux. Arch. EntwMech. Org.,' vol. **128**, p. 564.
—— (1933, *b*). 'Naturwiss.,' vol. **21**, p. 861.
Buddenbrock, W. von (1930). 'Z. Morphol. Okol.,' vol. **18**, p. 701.
—— (1931). 'Z. vergl. Physiol.,' vol. **14**, p. 415.
Bytinski-Salz, H. (1933). 'Roux. Arch. EntwMech. Org.,' vol. **129**, p. 356.

Dewitz, J. (1902). 'C. R. Soc. Biol. Paris,' vol. **54**, p. 44.
—— (1924). 'Zool. Jahrb. Abt. Allg. Zool. Phys.,' vol. **41**, p. 245.
Fraenkel, G. (1934). 'Nature,' vol. **133**, p. 834, June 2.
Hewitt, C. G. (1914). "The House-Fly," Cambridge.
Hopp, M. (1933). 'Zool. Jahrb. Abt. Anat. Ont.,' vol. **57**, p. 433.
Koller, G. (1929). 'Biol. Rev.,' vol. **4**, p. 269.
Kopeć, S. (1922). 'Biol. Bull.,' vol. **42**, p. 323.
—— (1924). 'Biol. Bull.,' vol. **46**, p. 1.
Lowne, B. T. (1890–92). 'The Blow-Fly,' vol. **1**, London.
Wardle (1930). 'Ann. app. Biol.,' vol. **17**, p. 568.
Wigglesworth, V. B. (1934, *a*). 'Nature,' vol. **133**, p. 725.
—— (1934, *b*). 'Quart. J. Micr. Sci.,' vol. **77**, p. 191.

Explanation of Plate 1

All the animals are orientated with the front end towards the top of the page.

Fig. 1—Both parts pupate within 16 hours after ligaturing.

Fig. 2—The hind part alone pupates within 16 hours after ligaturing.

Fig. 3—The front part alone pupates after more than 16 hours from ligaturing.

Figs. 5 and 6—Ligature laid down in front of the ganglion.

Fig. 5—Both parts pupate within 16 hours after ligaturing.

Fig. 6—Hind part alone pupates more than 16 hours after ligaturing.

Fig. 7—The whole body except the very front end immersed in oil during the colouring of the pupa: colouring starts on the front end and spreads gradually backward.

Fig. 8—Middle part of a twice-ligatured mature lava remains unpupated.

FIG. 1

FIG. 2

FIG. 3

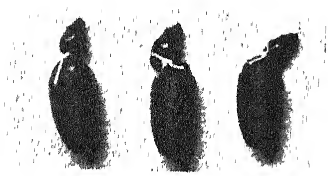


FIG. 5

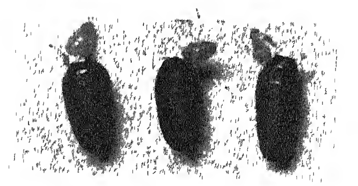


FIG. 6

FIG. 7

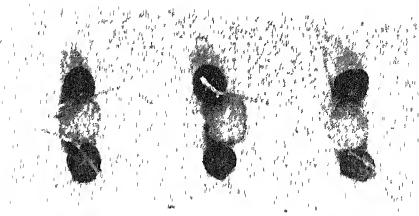


FIG. 8

(*Facing p.* 12)

612.662.599.8

The Menstrual Cycle of the Primates
VIII—The Œstrin-Withdrawal Theory of Menstruation

By S. ZUCKERMAN*

(From the Departments of Physiology and Obstetrics, Yale University School of Medicine)

(*Communicated by Sir Grafton Elliot Smith, F.R.S.—Received February* 19, 1935)

1—INTRODUCTION

In spite of ample evidence that œstrin is not the sole endocrine factor directly concerned in the changes which take place in the uterus, the hypothesis that normal menstruation is essentially a katabolic process following a temporary cessation of œstrin activity, still finds acceptance (*e.g.*, De Jongh and Laqueur, 1931; Allen, 1932). This hypothesis, formulated originally by Allen (1927), is primarily based upon three sets of well-established observations on monkeys.† These are: (*a*) uterine bleeding follows soon after bilateral, sometimes after unilateral, ovariectomy; (*b*) uterine bleeding may be precipitated by injury to large or cystic ovarian follicles; and (*c*) uterine bleeding almost always begins a few days after the cessation of injection of œstrin into both normal and castrate monkeys. Corresponding observations have also been reported for man. Thus, the first is a clinical fact which had been recognized long before monkeys were used experimentally in studies of the reproductive processes. The same applies to the second; for example, as long ago as 1890 Cohnstein reported that menstruation is precipitated by manual pressure of the ovaries. The third finding has also been recently shown to be true for man; thus Kaufmann (1934) found that uterine bleeding occurs in women after the administration of 1,000,000 international units of œstrin. In spite of its apparently solid foundation, the œstrin-withdrawal theory of menstruation nevertheless falls short as an adequate statement because of its failure to account for certain essential facts. These deficiencies are discussed in the conclusion to this paper. Regarded purely as a working hypothesis, however, the

* At the time of this investigation a Rockefeller Research Fellow.

† For references to the literature, *see* Allen (1932).

theory immediately suggests that menstruation would be completely inhibited or delayed by the administration of œstrin previous to the expected onset of uterine bleeding. This deduction has not been systematically tested. The following are the only observations bearing on the question that I have been able to find. (*a*) Kurzrok (1932) has reported that too prolonged injections of œstrin may completely stop menstruation in young patients with oligomenorrhœa; (*b*) Hartman (1934) has described an experiment in which a monkey was given 995 rat units of œstrin over a period of 8 days, beginning on the 18th day of a cycle. Menstruation began during the course of injections, at approximately the time that it would have occurred if œstrin had not been given, and demarcated a cycle of 24 days. Unfortunately, conclusions cannot be drawn from either of these observations, although what both Kurzrok and Hartman did is essentially what should be done if the œstrin-withdrawl hypothesis of menstruation is to be critically tested.

The experiments described in this paper, carried out in 1933 and 1934, aimed at providing such a test. Attempts were made, by injecting keto-hydroxy-œstrin (œstrone), to influence the rhythm of successive cycles of two regularly menstruating monkeys.

2—Experimental

(*a*) *Sooty Mangabey*

The first set of experiments was performed on a mature and healthy Sooty Mangabey (*Cercocebus torquatus atys*) whose weight varied around 7 kg. The animal had given birth to a baby on January 26, 1933. Her first post-partum bleeding began on January 30—2 days after her baby's death. The succeeding menstrual period began on March 21 and demarcated a post-partum cycle of 50 days. The ensuing three cycles were respectively 30, 31 and 30 days long. Although not so regular as those of a baboon, the animal's phases of sexual-skin swelling were nevertheless sufficiently well-defined to show that subsidence of swelling began at varying times between approximately the 14th and 20th days of the cycle.

Œstrin was administered daily in increasing amounts in the subsequent eight cycles, beginning at different times before the expected onset of menstruation, and, except in the last cycle, continuing until bleeding began. Both aqueous and oily preparations were injected. The relevant data are tabulated in Table I.

TABLE I—EFFECT OF ŒSTRIN ON LENGTH OF CYCLE IN A SOOTY MANGABEY

No. of cycle	Length of cycle, days	Injections begun, day of cycle	Œstrin injected, rat units*
1	50†	—	—
2	30	—	—
3	31	—	—
4	30	—	—
5	29	29	200
6	30	24	1,500
7	31	22	2,800
8	30	23	3,500
9	36	20	11,850
10	32	20	9,000
11	31	18	10,750
12	55‡	12	33,675

* According to the Report of the International Committee for the Standardization of œstrin, 1 rat unit, as assayed by the method of Doisy, is approximately equivalent to 3 International units.

† Post-partum.

‡ Animal died before the onset of menstruation.

Doses—As a rule courses of injections began with 250 rat units a day and worked up to a daily maximum of 1000 rat units. At the end of the final series (12th cycle), 2000 rat units were being given daily.

Sexual Skin—The normal sexual-skin rhythm was not disturbed in cycles 5, 6, and 7. In the 8th cycle, the sexual skin underwent a normal cycle of activity, but about a week after its subsidence (*i.e.*, on the third day of injections—the 25th day of the cycle) it began to swell again. On the last day of this cycle swelling and tension were at their maximum.

From this time onwards both the normal sexual-skin cycle and the usual response to œstrin injections were disturbed. For example, in the 9th cycle, swelling did not definitely begin until 12 days after the beginning of injections. Similarly, there was no sign of sexual-skin activity during the first 20 days of the 10th cycle, when œstrin was not being administered, but swelling began on the 3rd day of injections (the 22nd day of the cycle), only to subside about 10 days later, while injections were still being given.

The nipples showed changes which closely paralleled those of the sexual skin.

Uterine Bleeding—Menstruation was usually more prolonged than normal, the mean duration being approximately 5 days (range 3–8 days).

The 12th Cycle—On March 6, the 41st day of the 12th cycle, the animal suddenly and for the first time showed signs of illness. From that day she grew steadily worse, until her death on March 18. Her illness was marked by an obvious anæmia, by widespread subcutaneous hæmorrhages, and by bleeding from the anus and nostrils. There was no sepsis at any site of injection, and body-weight fell but slightly.

Œstrin injections were stopped on March 14, the 50th day of the cycle, and on March 18 the animal died. There was no indication in the autopsy findings that her death was either caused or hastened by the injections.

The ovaries were fixed in Bouin's fluid and stained with hæmalum and eosin. The fixed and stained left ovary measures 9·6 × 7 × 4 mm, the right 8·85 × 7 × 3·5 mm. Primordial follicles are much reduced, presumably in part as a result of the prolonged œstrin treatment (*see* Allen, 1928), and atretic follicles are exceeding numerous. No normal follicle has a diameter of more than 1 mm. The left ovary contains the remains of three corpora lutea, whose staining reactions are indicative of their different ages. The right ovary, whose follicular picture is the same as that of its fellow, contains the remains of two other corpora lutea. None of these five corpora has the characteristics of one that would have developed as recently as during the animal's last cycle, and in view of what is known of the length of time that a corpus luteum can persist in the ovary as a recognizable structure, it is safe to assume that the five corpora relate to the animal's five preceding cycles.

(*b*) *Common Macaque*

The animal investigated was a reproductively mature, though not quite adult, specimen of *Macaca irus*, whose weight varied in the region of 3·1 kg. A course of œstrin injections was begun 18 days after the purchase of the animal, and before menstruation had been observed. Injections were continued for 7 days, 1800 rat units being given. On the 8th day uterine bleeding began and continued for 3 days. Injections were stopped on the 1st day of bleeding.

On the 13th day of the cycle begun by this bleeding, injections were once again started, and continued for 17 days, a total of 9750 rat units being administered. Uterine bleeding began on the 17th day of injections, demarcating an interval between the two bleedings of 28 days. Bleeding lasted 5 days.

The animal was left undisturbed during her third cycle, which lasted 35 days. On the 8th day of her fourth cycle she was castrated. Uterine

bleeding began 8 days after the operation, and continued on and off for about 50 days. The ovaries were treated in the same way as those of the Mangabey.

The right ovary contains an almost mature follicle. In it are also imbedded three old corpora lutea exhibiting, as is usual, different staining reactions indicative of their different ages. The left ovary contains a large and fairly recent corpus luteum, which fills practically the entire organ. Presumably it dates from the non-experimental cycle immediately preceding the cycle in which the animal was castrated, and is thus about three weeks old. In addition, this ovary also contains the remains of older corpora lutea. In all 6 recognizable corpora lutea are present in the ovaries of this animal, and it is therefore fairly safe to assume that the animal had ovulated regularly in the 6 cycles preceding her castration.

Discussion

After the cessation of œstrin injections into castrate monkeys, a latent period intervenes before the onset of uterine bleeding. Allen (1927) found this interval to be 3–7 days; Smith and Engle (1932) state it to be 6; the average latency in 10 experiments of my own was 9·2 (range 5–14 days). So far as the cessation of injections is the significant factor in the precipitation of uterine bleeding in castrate monkeys (in whom bleeding would not otherwise occur), the occurrence of this latent period naturally suggests that the actual stimulus to endometrial destruction begins to be effective about a week before bleeding begins. The view that normal menstruation is also due to a temporary cessation of œstrin activity thus implies that the amount of the hormone available in the body begins to be reduced about a week before the onset of bleeding. It is conceivable that once this stimulus of œstrin reduction has operated, the subsequent administration of the hormone is unable to inhibit the effect of its previous withdrawal. Consequently it might be argued that œstrin given in the last week of the menstrual cycle would be unable to delay menstruation, even though uterine bleeding is in fact due to a lack of the hormone.

If the œstrin-withdrawal theory of menstruation is sound, œstrin should, however, be able to inhibit menstruation if its administration were begun more than a week before the expected onset of bleeding. Actually the data reported in this paper show that this condition is not borne out in practice. Excluding the last cycle of the Mangabey, which is discussed below, there are 9 cycles in which the experiment was

tried. In 8 of these the administration of œstrin was begun 7 or more days before the onset of menstruation (mean, 12 days before, range 7–17 days). None of the 9 cycles was unduly prolonged (mean duration 31 days, range 28–36 days).

These data show that the administration of œstrin in the later half of the cycles does not affect the cycle's duration. The argument may, however, be raised that an inadequate amount of the hormone was injected in the present series of experiments, and that the conclusion is consequently unwarranted. Available evidence does not support this objection. Hisaw (1933) has found that as few as 50 rat units of œstrin daily will maintain the endometrium of a castrated monkey in a sufficiently functional condition for uterine bleeding to occur when the daily dose is reduced to 25 rat units. In the present series of injections the minimum daily dose was many times greater than this threshold dose, the average being in the neighbourhood of 500 rat units. The conclusion that has been drawn does not therefore appear to be ill-founded so far as this condition is concerned.

Ovulation in the baboon (Zuckerman, 1930; Zuckerman and Parkes, 1932) and in the Rhesus macaque (Hartman, 1932) generally occurs about the middle of the cycle, and data considered elsewhere (Zuckerman, 1930) suggest that in this respect these more closely studied animals are expressing a rule that applies generally to the Old-World primates that have been under investigation. In view of the histological evidence given above, it would thus follow that, with the exception of the last cycle of the Mangabey, ovulation had already occurred in each of the present experimental series of cycles before injections were begun. The experimental data under discussion may therefore be taken to indicate that the injection of keto-hydroxy-œstrin after ovulation does not delay the appearance of the next menstrual bleeding.

On the basis of this finding, one may consider the excessive length of the Mangabey's last cycle (55 days), in which injections were begun well before its mid-point, and in which ovulation did not occur. Histological examination showed that the animal's endometrium at the close of this cycle's course of injections was in a condition of cystic hyperplasia (Zuckerman and Morse, 1935). It is beginning to be recognized that this is the usual endometrial reaction to prolonged unopposed activity of œstrin (*see*, for example, Burch and his co-workers, 1932; Kaufmann, 1934). The condition of the Mangabey's uterus thus finds some explanation in the absence of a corpus luteum during the long period œstrin was being injected. The fact that the endometrium was capable of reacting in the way it did, suggests strongly that the cycle's prolongation,

at least in the initial phases, was due to the œstrin and not to the illness which was first manifested on the 41st day. The non-occurrence of ovulation in this cycle may conceivably have been an indirect effect of the action of the injected œstrin on the anterior lobe of the pituitary (*see* Meyer and his co-workers, 1932; Moore and Price, 1930; del Castillo, 1932). On the other hand, it is also possible that the cycle would have been an ovulatory one, such as sometimes occurs in the Rhesus monkey (Corner, 1923; Hartman, 1932), whether or not œstrin had been injected.

In the first seven experimental cycles of the Mangabey, and in the two of the Common Macaque, the action of the injected œstrin was opposed by that of endogenous luteal secretion. Available data show that the effects of the luteal secretions override those of œstrin. Thus Smith and Smith (1931) have found that in rabbits the injection of progestin stimulates the excretion of injected œstrin. An investigation of Smith and Engle (1932) is even more convincing. These investigators found that progestin injections, begun in monkeys immediately after the close of a course of œstrin and anterior pituitary injections, delays the uterine bleeding that would otherwise occur, bleeding being postponed until after the cessation of the progestin treatment. This finding indicates clearly that so far as the monkey's uterus is concerned the luteal is dominant over the follicular hormone. The failure of œstrin to inhibit menstruation when administered after ovulation can thus be explained as due to the inability of the hormone to alter the course of the endometrial cycle during the post-ovulatory phase, when the uterus is more specifically under the influence of the luteal secretions.

In the present state of our knowledge, it is of little use to argue that any except ovarian factors are immediately and directly concerned in the process of menstruation. The attempt of Hartman and his co-workers (1930) to ascribe menstruation to the effect of a specific hypophyseal secretion has not been sustained, and attempts to corroborate their findings have not been successful (*e.g.*, Saiki, 1932; Bachman and co-workers, 1935). In this connection it may also be noted that investigations of non-primate mammals, for example the ferret, have shown that œstrin is fully capable of exercising its uterine effects in hypophysectomized animals, a finding which is at variance with that of Hartman on monkeys (*see*, for example, Smith, P. E., 1932; Hill and Parkes, 1933).

So far as ovarian factors are concerned, the results of the present investigation indicate plainly that the œstrin-withdrawal theory of menstruation, though a possible interpretation of the uterine bleeding that occurs in non-ovulatory cycles, is an inadequate explanation of the

menstruation which terminates ovulatory cycles. It is inadequate because it does not take into account the action of the secretions of the corpus luteum.

I wish to express my thanks to Dr. Gregory Stragnell of the Schering Corporation, and to Dr. Oliver Kamm of the Parke Davis Company, for their kindness in supplying me with the œstrin (theelin and progynon) used in this study. To Dr. G. Van Wagenen I am deeply indebted for her gracious facilitation of the work. Part of the cost of the animals was defrayed out of a grant generously provided by the Medical Research Council.

4—Summary

Beginning at different times in the later half of the menstrual cycle, and before the expected onset of menstruation, œstrone (keto-hydroxy-œstrin) was injected daily, until uterine bleeding occurred, during seven cycles of a Mangabey, and two of a Common Macaque monkey. The average daily dose was approximately 500 rat units, the average total amount injected 5700 rat units. The injection did not disturb the normal menstrual rhythm (average duration of experimental cycles 31 days, range 28–36). Histological study provided evidence that ovulation had occurred in each of these experimental cycles.

Injections in the last cycle of the Mangabey were begun before what would have been its normal mid-point, and continued until the 50th day of the cycle. The animal suddenly sickened about a week before injections were stopped; it died four days following the last injection. A total of 33,675 rat units of œstrone was injected. Histological examination showed that ovulation had not occurred.

The relation of these findings to the œstrin-withdrawal theory of menstruation is discussed.

References

Allen, E. (1927). 'Contr. Embryol.,' Washington, vol. **19**, p. 1.

—— (1928). 'J. Morph. Physiol.,' vol. **46**, p. 473.

—— (1932). "Sex and Internal Secretions," Williams & Wilkins, Baltimore.

Bachman, C., Collip, J. B., and Selye, H. (1935). 'Proc. Roy. Soc.,' B, vol. **117**, p. 16.

Burch, J. C., Wolfe, J. M., and Cunningham, R. S. (1932). 'Endocrinology,' vol. **16**, p. 541.

Castillo, E. B. del (1932). 'Rev. soc. argent. Biol.,' vol. 8, p. 591.

Cohnstein (1890). 'Deuts. med. Wschr.,' vol. **16**, p. 764.

Corner, G. W. (1923). 'Contr. Embryol.,' Washington, vol. **15**, p. 75.
De Jongh, S. E., and Laqueur, E. (1931). 'Acta. Brev. Neerland.,' vol. **1**, p. 35.
Hartman, C. G. (1932). 'Contr. Embryol.,' Washington, No. 134, p. 1.
—— (1934). 'Amer. J. Obstet. Gynec.,' vol. **27**, p. 564.
Hartman, C. G., Firor, W. M., and Geiling, E. M. K. (1930). 'Amer. J. Physiol.,' vol. **95**, p. 662.
Hill, M., and Parkes, A. S. (1933). 'Proc. Roy. Soc.,' B, vol. **113**, p. 541.
Hisaw, F. L. (1932). "Sex and Internal Secretions," edited by E. Allen, Williams and Wilkins, Baltimore.
—— (1933). Quoted by G. W. Corner, 'Medicine,' vol. **12**, p. 61.
Kaufmann, C. (1934). 'Proc. R. Soc. Med.,' vol. **27**, ii, p. 849.
Kurzrok, R. (1932). 'Endocrinology,' vol. **16**, p. 361.
Meyer, R. K., Leonard, S. L., Hisaw, F. L., and Martin, S. J. (1932). 'Endocrinology,' vol. **16**, p. 655.
Moore, C. R., and Price, D. (1930). 'Proc. Soc. Exp. Biol.,' N.Y., vol. **28**, p. 38.
Saiki, S. (1932). 'Amer. J. Physiol.,' vol. **100**, p. 8.
Smith, P. E. (1932). 'Amer. J. Physiol.,' vol. **99**, p. 349.
Smith, P. E., and Engle, E. T. (1932). 'Proc. Soc. Exp. Biol.,' N.Y., vol. **29**, p. 1225.
Smith, G. van S., and Smith, O. W. (1931). 'Amer. J. Physiol.,' vol. **98**, p. 578.
Zuckerman, S. (1930). 'Proc. Zool. Soc.,' London, p. 691.
Zuckerman, S., and Morse, A. W. (1935). 'Surg. Gynec. Obstet.,' (*in the press*).
Zuckerman, S., and Parkes, A. S. (1932). 'Proc. Zool. Soc.,' London, p. 139.

612 . 662 : 599 . 8

The Menstrual Cycle of the Primates
IX—The Effect of Œstrin on the Denervated Sexual Skin

By S. ZUCKERMAN*

(Departments of Physiology and Obstetrics, Yale University School of Medicine)

(*Communicated by Sir Grafton Elliot Smith, F.R.S.—Received February* 19, 1935)

1—INTRODUCTION

Before the demonstration of the part played by hormones in the behaviour of the reproductive organs, many efforts had been made to discover ways in which neural influences are concerned in generative mechanisms (*see*, for example, Budge, 1858; Simpson, 1871; Goltz, 1874; Goltz and Ewald, 1896; Rein, 1880; Ribbert, 1898; Mironoff, 1895; also early work on ovarian transplantation, *e.g.*, Halban, 1901). The general conclusion derived from these earlier studies was that the integrity of the nerves of the reproductive organs is not essential for conception, gestation, parturition, and lactation.

The question has been reconsidered more recently, with the same general result. Thus Sweet and Thorp (1929), Cannon and his co-workers (1929, 1931), and Bacq (1931, 1932) have found that total or abdominal sympathectomy in dogs, cats, and rats neither disturbs the periodicity of the œstrous cycle, nor prevents fecundation and parturition. The chief ill-effect of total sympathectomy, so far as the reproductive functions are concerned, is impaired milk secretion. Bacq, Brouha and Hinglais (1932) have also shown that œstrin and the gonadotropic hormones of the anterior lobe of the pituitary can exercise their normal effects upon the uterus and ovaries of rats, cats, rabbits, and guinea pigs following removal of the lumbar sympathetic chains and the mesenteric plexuses.

Evidence is, however, available to show that neural lesions disturb the rhythm of the menstrual cycle in monkeys (van Wagenen, 1933; Zuckerman, 1934). Moreover, it has been shown (Bouin and Courrier, 1929; Bucheim and Zaleski, 1932) that division of the cervical sympathetics of rabbits intensifies the changes which in an aural transplant of

* At the time of the investigation, a Rockefeller Research Fellow.

a uterine horn normally parallel the changes occurring in the uterine horn still *in situ* in the pelvis. To throw more light on the general question of neural influences in generative mechanisms, an effort was therefore made to discover if the changes which occur in the sexual skin of monkeys following the administration of œstrin are the same after denervation of the region concerned as in the intact animal.

FIG. 1—Rhesus monkey (142) showing quiescent sexual skin previous to denervation. Approx. × $\frac{1}{3}$

FIG. 2—Swollen and active sexual skin of Rhesus monkey (142) following denervation and in response to injections of œstrone. Approx. × $\frac{1}{2}$

2—METHODS AND MATERIAL

Three Rhesus Macaques (*Macaca mulatta*) and one pig-tailed Macaque (*Macaca nemestrina*) were used in the present study.

The sexual-skin response of the Rhesus monkey has been frequently described, and comprises reddening, with some swelling, of an extensive circum-genital area which may pass forward on to the symphysis pubis, posteriorly to the base of the tail and the back, laterally to the flanks, and downward as far as the popliteal fossæ, fig. 2.

The sexual skin of the pig-tailed Macaque is not so well known. The skin ventral to the pudendal cleft swells into an elongated pubic lobe which includes the clitoris. The anterior limit of this part of the swelling is concealed slightly by abdominal hair. The pudendal margins and the skin surrounding the anus swell into a prominent, smooth, blue-red anal pad which extends laterally round the callosities, which are neither affected themselves nor covered by surrounding swelling. The "circum-anal pad" may extend upward to involve the base of the tail, so that the tail becomes more fixed than it is otherwise. In its fully swollen condition the sexual skin as it were caps the hinder end of the animal's trunk like the head of a mushroom, a deep cleft being formed between the swollen and the unaffected areas, figs. 4 and 5.

Innervation of the Sexual Skin—The combined sensory fields of the 8th, 9th, and 10th post-thoracic nerves of the Rhesus monkey (1st, 2nd, and 3rd sacral nerves) correspond very closely with the normal extent of the sexual skin (Sherrington, 1892, 1893). The pilo-motor nerves to the area pass down the lumbar sympathetic chains from the 12th thoracic and 1st, 2nd, and 3rd lumbar nerves (Langley and Sherrington, 1891). The vaso-constrictor fibres to the sexual skin region are also part of the lower thoraco-lumbar spinal sympathetic outflow, and thus they too pass through the lumbar sympathetic chains on their way to the sacral plexus (Langley, 1900). There are several possibilities so far as vaso-dilator nerves of the sexual skin are concerned: (*a*) the afferent nerves of the area may transmit antidromic vaso-dilator impulses; (*b*) some of the parasympathetic fibres that emerge from the cord with the upper sacral nerves may reach the blood vessels of the sexual skin, particularly those in the near vicinity of the pudendal cleft (Langley and Anderson, 1895); (*c*) vaso-dilator impulses may be transmitted by the sympathetic nerves; (*d*) if certain views that have been recently expressed are correct (*e.g.*, Bishop and co-workers, 1933; Kahr and Sheehan, 1933; Kuré and co-workers, 1930), efferent impulses concerned in vaso-dilatation are transmitted along the posterior roots of all segments of the spinal cord. If such a mechanism operates in the sexual skin, the channels along which these vaso-dilator impulses pass would be the afferent nerves of the area—the 1st, 2nd, and 3rd sacral nerves. Whether or not future work establishes the existence of this last vaso-dilator mechanism, it follows that denervation of the sexual skin of the Rhesus Macaque can be completely effected by division of the lumbar sympathetic chains and the sacral nerves.

The disposition of the peripheral somatic and autonomic nerves in the

pig-tailed monkey is described elsewhere (Zuckerman, 1935). Its anatomy appears to be identical with that of the Rhesus monkey.

Some of the animals used in this study had previously been used in another investigation, and some of the operative procedures necessary for denervation of the sexual skin area had already been performed. Additional operations, such as division of the hypogastric nerves, chemical denervation of the ovaries, castration, evulsion of the lumbar sympathetic chains, transection of the spinal cord, had also been performed in a few cases. None of these collateral operations in any way affected the problem of the present investigation, except that they added to the extent of denervation of the reproductive organs.

All operations were carried out with sterile precautions under amytal or nembutal anæsthesia. Lesions were later verified at autopsy. The precise operations performed on each animal are mentioned below.

FIG. 3 — Photograph showing the active sexual-skin area of Rhesus monkey (142), following denervation, extending along the tail and up the back

3—EXPERIMENTAL

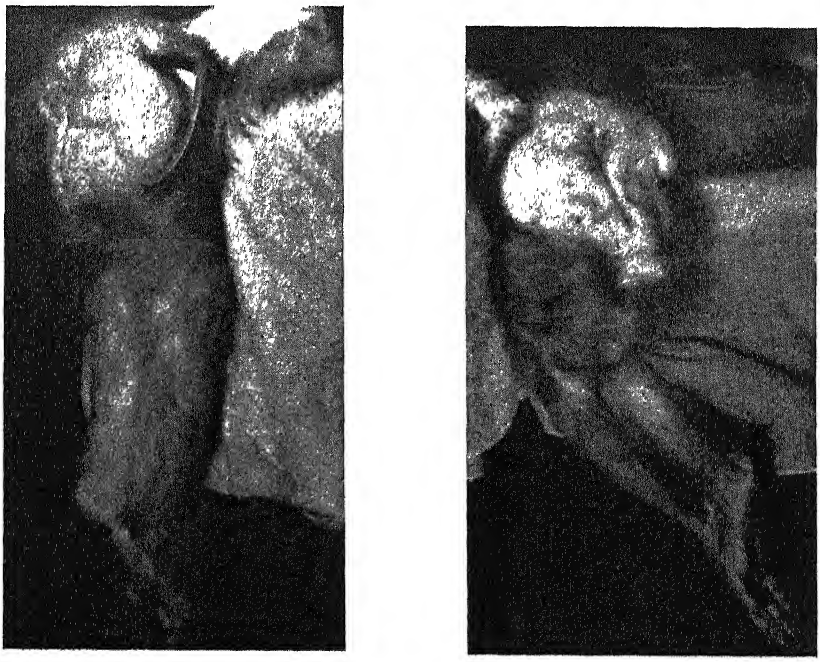
Experiment 1—Rhesus monkey, *Macaca mulatta* 142. Sub-adult, but reproductively mature. Body-weight 3750 gm.

Previous to her use in this experiment, the following operations had been performed on this animal: (*a*) bilateral gonadectomy, (*b*) extradural division of the right 12th thoracic, 1st, 2nd, 3rd, and 4th lumbar nerves, (*c*) bilateral division of the splanchnic nerves, (*d*) bilateral evulsion of the lumbar sympathetic chains.

The day the animal was first used in the present experiment her sexual skin showed little colour and practically no swelling, fig. 1. On that day, the spinal canal was opened and the cauda equina transected so as to divide the 6th lumbar nerves and all the nerves below them. Immediately after the operation a course of keto-hydroxy-œstrin (œstrone) injections

was begun. Injections were given once daily for 11 days, a total of 8500 rat units being administered.

The sexual skin began to change colour the day after the first injection was given. It soon started swelling and at the end of the course the swelling had assumed considerable proportions for a Rhesus monkey, figs. 2 and 3. The swelling extended on to the back, the skin over the iliac bones and sacrum being thrown into a series of vertical folds. The base of the tail, both dorsally and ventrally, was also swollen. The swelling extended laterally on to the flanks and downward to the popliteal



Figs. 4 and 5—Photographs showing active and swollen sexual skin of normal pig-tailed Macaque. Approx. $\times \frac{1}{6}$

fossæ, the skin on the buttocks and thighs being corrugated transversely. The " scrotal folds " showed little swelling, but there was a prominent mid-line roll of swelling connecting them with the sheath of the clitoris. The lips of the pudendal cleft became very tense and swollen.

The sexual skin remained a deeper and more purplish red than usual throughout the course of injections, at no time assuming the bright red colour which often occurs in Rhesus monkeys.

As usually happens, the nipples responded to the œstrin injections by becoming more highly coloured and prominent.

It is of interest that the hairs of the denervated swollen sexual skin were erect in the way they are in the active sexual skin of the normal animal. This fact is discussed below.

Experiment 2—Pig-tailed Macaque, *Macaca nemestrina.* Mature female, body-weight 4300 gm.

The animal had been under observation for several months before being used in this experiment. Her spinal canal was opened and the cauda equina transected, so as to divide the 7th lumbar and all the sacral

FIG. 6—Photograph of inactive and collapsed sexual skin of pig-tailed Macaque

FIG. 7—Photograph showing swollen sexual skin of pig-tailed Macaque after denervation, and in response to œstrone. The dark areas are trophic ulcers

nerves, on a day when her sexual skin was fully swollen (May 22, 1934), figs. 4 and 5. The day following the operation the swelling of the sexual skin began to decline. A week later both lumbar sympathetic chains were divided by the transperitoneal route, small segments, including the 6th lumbar ganglia, being removed. At the time of this second operation, the sexual skin swelling had been completely absorbed, the pubic lobe and circum-anal pad being at their resting level, and the tail no longer fixed, fig. 6.

Renewed signs of sexual-skin activity were first noticed on June 12,

about a fortnight later. By June 18 the sexual skin was moderately swollen and a bright deep red in colour.

Œstrone injections were given between June 18 and June 25, a total of 7500 rat units being injected. At the end of this course of injections, the sexual skin, with the exception of the ventral part of the pubic lobe, was fully expanded, fig. 7. Its colour, however, was paler than it was before injections were begun. As fig. 7 shows, the tail was once again fixed owing to swelling of its base.

The fact that the pubic lobe of the sexual skin was not fully swollen cannot be regarded as significant, since the experiment had to be summarily ended after only one week of injections, and while the sexual skin was still expanding.

The animal's normal nipples, as well as an accessory nipple situated on the right side, became highly coloured and swollen during the course of injections. The hairs on the swollen denervated sexual skin stood out as erect as they previously had on the normal swelling.

"Trophic ulcers" developed on the deafferented sexual skin in the region of the callosities.

In the following experiments only part of the procedures necessary for complete denervation of the sexual skin was carried out, but the results obtained throw some light on the influence of nervous factors in the sexual-skin mechanism.

Experiment 3—Rhesus monkey, *Macaca mulatta* Sg. A reproductively mature animal, but immature as judged by dentition. Body-weight 4650 gm.

Beginning on the eighth day of a menstrual cycle, the animal was given a course of daily œstrone injections over a period of 15 days (April 30 to May 14) until a total of 5625 rat units had been administered. On the third day of injections, May 2, the spinal canal was opened and the cauda equina divided so as to sever all the sacral nerves.

The first definite sign of a sexual-skin response—reddening—was seen on May 4, the fifth day of injections, and the second day after operation. The response remained poor, and at the end of the course of injections there was practically no swelling and the colour was only of medium intensity.

The above operation had presumably interfered only with the vaso-dilator mechanism of the sexual skin, the lumbar sympathetic chains being left intact. At a later stage further operations were carried out, but again none involved division of the post- or pre-ganglionic pathways of the sexual skin's vaso-constrictor nerves. Subsequently a second series

of œstrone injections was given, a total of 8500 rat units being injected over 11 days. The sexual-skin response was not appreciably better than it had been during and after the first course of injections.

Experiment 4—Rhesus monkey, *Macaca mulatta* B.C. Body-weight 4095 gm. A reproductively mature Rhesus monkey, immature on the criterion of dental age.

This experiment was the converse of the previous one, the vaso-constrictor pathways to the sexual skin being divided, and the vaso-dilator channels being left mostly undisturbed.

The first operation performed on this animal was extraperitoneal division of the nervi erigentes. So far as is definitely known, this procedure would at most interfere with the vaso-dilator mechanism in the close neighbourhood of the pudendal cleft. Following this operation, the animal was subjected to a course of œstrone injections (3000 rat units over a period of 7 days). The response was pronounced and completely normal.

Fig. 8—Inactive sexual skin of Rhesus monkey B.C. previous to operation

At the end of this course of injections the lumbar sympathetic chains and the hypogastric nerves were divided, thus interrupting the pilomotor and vaso-constrictor pathway to the sexual skin. A fortnight after this operation the sexual skin was altogether quiescent, fig. 8. A second course of œstrone injections was then begun, 8750 rat units being injected over a period of 12 days. The response was immediate and even more conspicuous than that which attended the first course of injections. The sexual skin became highly coloured and very swollen. Transverse corrugations appeared on the animal's hindquarters, and swelling extended to the base of the tail and to the back. The most conspicuous effect was produced in the skin ventral to the symphysis pubis. The hairs of the sexual skin were as erect in the partially denervated active sexual skin as they are during normal phases of sexual-skin swelling, figs. 9 and 10.

4—Discussion

The first two experiments described above show plainly that œstrin acts as readily and as well on the denervated as on the normal sexual skin.

Particular significance cannot be attached to the deep colour of the skin in the first experiment, since this hue is sometimes exhibited by normal Rhesus monkeys during active phases of their sexual skin cycles.

Histological study suggests that the increase in coloration of the sexual skin during phases of activity is due to dilatation and engorgement of the vessels beneath the epithelium. According to Collings (1926) there is a direct correlation between the degree of reddening on the one hand and the size of the superficial vessels and the degree of engorgement on the other. The swelling is mainly due to an œdema of the subcutaneous

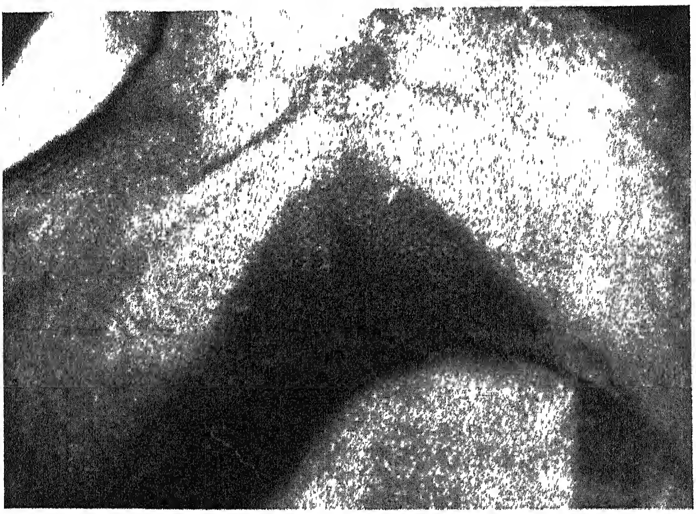


FIG. 9—Active sexual skin of Rhesus monkey B.C., after division of the lumbar sympathetic chains, showing the highly coloured area anterior to the symphysis pubis

tissues (Parkes and Zuckerman, 1931). Whatever be the immediate cause of the œdema, it is plain that the primary change in the sexual skin in response to œstrin is a vascular one. Consequently it can be inferred that œstrin may act directly upon the blood vessels.

This does not necessarily mean that the nerves of the sexual skin and its vessels exert no influence in the sexual skin mechanism. In the normal animal there is conceivably a physiological balance between sympathetic and parasympathetic control of at least the central sexual-skin vessels. Similarly in the completely denervated preparation there is a parallel loss of both these controls. In both these cases, the sexual-skin response could be regarded from the point of view of nervous factors as a balanced one. On the other hand, if one side of the autonomic mechanism suffici-

ently overpowered the other, the manifestations of sexual-skin activity might be different from those which would appear were the other side dominant. This indeed is the conclusion to which the results of experiments 3 and 4 may tentatively be regarded as pointing. In experiment 3, the pelvic vaso-dilator nervous mechanism was presumably disturbed, the vaso-constrictor nervous mechanism being intact. The sexual-skin response in this experiment was poor. The reverse conditions held in experiment 4, and the sexual-skin response (*i.e.*, vaso-dilatation, etc.) was excellent. The whole question needs closer investigation before any definite conclusions are drawn.

In experiments 1, 2, and 4 the lumbar sympathetic chains had been divided and the pilo-motor pathways to the sexual skin consequently interrupted. In spite of this, the hairs became erect when the sexual skin expanded in response to injections of œstrin. It is therefore likely that the erection of hairs which normally accompanies sexual-skin activity in intact animals is due, not to centrally discharged pilo-motor impulses, but either to direct pull upon the hair follicles occasioned by œdema, or to local stimulation of the pilo-motor nerve-endings by the increased tension of the subcutaneous tissues.

FIG. 10 — Active sexual skin of perineal region and base of tail of Rhesus monkey B.C., following division of the lumbar sympathetic chains

I wish to express my indebtedness to Dr. Oliver Kamm of the Parke Davis Company, and to Dr. Gregory Stragnell of the Schering Corporation for the supplies of œstrone (theelin and progynon) used in this study.

SUMMARY

The sexual skin of a castrated Rhesus Macaque was denervated by evulsion of its lumbar sympathetic chains and by bilateral division, in the spinal canal, of all nerves below and including the 6th lumbar. A course of œstrin injections was begun immediately after the latter operation. The response of the sexual skin did not differ in any significant way from that of the normal animal.

The experiment was repeated on a pig-tailed macaque, with the same result.

The sexual skin of a second Rhesus Macaque was partially denervated by bilateral division, within the spinal canal, of the sacral nerves, the lumber sympathetic chains being left intact. Subsequent œstrin injections induced only slight changes in the sexual skin.

The lumbar sympathetic chains and the nervi erigentes were divided in a third Rhesus monkey, the sacral nerves being left intact. Subsequent œstrin injections induced pronounced changes in the sexual skin.

It is concluded that œstrin may act directly on the blood vessels of the sexual skin. At the same time the possibility that the nerves of the vessels may also exert an influence in the sexual-skin mechanism is not excluded.

The course of the pilo-motor nerves to the sexual skin had been interrupted in the three preparations in which the lumbar sympathetic chains had been divided. Nevertheless, the hairs of the sexual skin stood out as they do in the normal active sexual skin during the experimental phases of œstrin activity following these operations. It is concluded that their erection in the normal animal may be a mechanical result of œdema of the subcutaneous tissue, and not an effect of centrally discharged pilo-motor impulses.

References

Bacq, Z. M. (1931). 'Endocrinology,' vol. **15**, p. 34.

—— (1932). 'Amer. J. Physiol.,' vol. **99**, p. 444.

Bacq, Z. M., Brouha, L., and Hinglais (1932). 'Gynéc. et Obstét.,' vol. **26**, p. 97.

Bishop, G. H., Heinbecker, P., and O'Leary, J. L. (1933). 'Amer. J. Physiol.,' vol. **106**, p. 647.

Bouin, P., and Courrier, R. (1929). 'Arch. Anat. Micr.,' Paris, vol. **25**, p. 189.

Buchheim, W., and Zaleski, W. (1932). 'C. R. Soc. Biol.,' Paris, vol. **104**, p. 896.

Budge, Professor (1858). 'Virchow's Arch.,' vol. **15**, p. 115.

Cannon, W. B., and Bright, E. M. (1931). 'Amer. J. Physiol.,' vol. **97**, p. 319.

Cannon, W. B., Newton, H. F., Bright, E. M., Menkin, V., and Moore, R. M. (1929). 'Amer. J. Physiol.,' vol. **89**, p. 84.

Collings, M. R. (1926). 'Anat. Rec.,' Philadelphia, vol. **33**, p. 271.

Goltz, F. (1874). 'Pflueger's Arch.,' vol. **9**, p. 552.

Goltz, F., and Ewald, J. R. (1896). 'Pflueger's Arch.,' vol. **63**, p. 362.

Halban, J. (1901). 'S.B. Akad. Wiss.,' Wien, vol. **110**, abth. 3, p. 71.

Kahr, S., and Sheehan, D. (1933). 'Brain,' vol. **56**, p. 265.

Kuré, K., Saégusa, G., Kawaguchi, K., and Shiraishi, K. (1930). 'Quart. J. Exp. Physiol.,' vol. **20**, p. 51.

Langley, J. N. (1900). In Schäfer's "Text-Book of Physiology," Edinburgh, Pentland, vol. **2**.

Langley, J. N., and Anderson, H. K. (1895). 'J. Physiol.,' vol. **19**, p. 85.

Langley, J. N., and Sherrington, C. S. (1891). 'J. Physiol.,' vol. **12**, p. 278.
Mironoff, M.M. (1895). 'Arch. sci. biol., St. Petersburg,' vol. **3**, p. 353.
Parkes, A. S., and Zuckerman, S. (1931). 'J. Anat.,' London, vol. **65**, p. 272.
Rein, G. (1880). 'Pflueger's Arch.,' vol. **23**, p. 68.
Ribbert, H. (1898). 'Arch. EntwMech. Org.,' vol. **7**, p. 688.
Sherrington, C. S. (1892). 'Proc. Roy. Soc.,' B, vol. **52**, p. 333.
—— (1893). 'Phil. Trans. Roy. Soc.,' B, vol. **184**, p. 641.
Simpson, J. Y. (1871). "Selected Obstetric Works," edited by W. H. Black, Edinburgh, Black.
Sweet, L. K., and Thorp, E. G. (1929). 'Amer. J. Physiol.,' vol. **89**, p. 50.
van Wagenen, G. (1933). 'Amer. J. Physiol.,' vol. **105**, p. 473.
Zuckerman, S. (1934). 'Anat. Rec.,' Philadelphia, vol. **58**, suppl. 43.
—— (1935). *In preparation.*

576.312.34
576.35

The Internal Mechanics of the Chromosomes
I—The Nuclear Cycle in *Fritillaria*

By C. D. Darlington, John Innes Horticultural Institution, Merton

(*Communicated by Sir Daniel Hall, F.R.S.—Received February* 2, 1935)

[Plate 2]

1—Introduction

The nuclear cycle in plants and animals consists in the alternation of two mechanically stable systems, the resting nucleus and the metaphase chromosomes. The regularity of this alternation and the constancy of the bodies taking part in it has led to the assumption that it depends on a "permanence" of the positional relationship of essential elements in these bodies, that is, on a property of passing on the same structure from mother to daughter nucleus and from mother to daughter organism by the structure reproducing its like without change. This assumption has been vindicated by the demonstration at a particular nuclear division, meiosis, in the transitional and generally unstable stages of prophase between the resting nucleus and the metaphase chromosomes, of a linear arrangement of particles which is constant both in individuals and in races. The inference of this constant arrangement of particles depends on evidence of form and of function. When the inference is from form the particles are *chromomeres*, when the inference is from function revealed by mutation, by crossing-over, or by specific attraction, the particles are *genes*.

The first problem in considering the nuclear cycle is therefore to discover the spatial relationship between the mechanically unstable prophase threads which reveal this exact and indisputable property of organization, viz., linear arrangement, and the stable resting nucleus and metaphase chromosomes, which are optically homogeneous in the living condition and whose properties of organization as revealed by treatment are disputable. We must find how the one changes into the other. The problem is dynamic not static. Its solution must depend on observations of the natural building up of the chromosomes in the prophase of mitosis, or of their natural or artificial breaking down in the telophase. It must be related to our knowledge of the permanence of the linear arrangement of particles at all stages and of their reproduction at one stage in the cycle. This study I would describe as the study of the *internal mechanics* of the chromosomes, that is of the principles governing their internal stresses and adjustments. I would thus distinguish changes within the chromosomes from the class of changes with which exact study has hitherto been chiefly concerned, namely, those such as pairing and terminalization, orientation, and disjunction, which affect the relationships of different chromosomes with one another and with other cell-elements. The principles governing these much simpler and clearer changes I would describe as the *external mechanics* of the chromosomes. The distinction between the two studies is arbitrary but convenient for analysis. They meet when we come to consider the mechanism of crossing-over. Together they must aim at explaining in common terms every change, no matter how trivial or how important, in the size, shape, position, and attitude that makes up the visible life of the chromosomes.

In order to observe and compare the arrangement of the permanent thread in the chromosomes and in the resting nucleus a change must be induced in both of them analogous to that which they undergo during the intermediate phases of the nuclear cycle, namely, separation into two phases by a redistribution of the water-content. A capacity for such a change is characteristic of protoplasmic systems. It may occur spontaneously or artificially, but when it occurs artificially we have no guarantee other than comparison that the structure observed is true to nature, is characteristic, that is to say, of the living structure. When we destroy the optical homogeneity we may destroy the spatial characteristics of the permanent organization. This indeed is what has usually happened in treatments of both the resting nucleus and the metaphase chromosomes. Consequently many conflicting observations of their structure have been made, and many dubious conclusions have been drawn with regard to their structural relationships. Even the time in the nuclear cycle when the

chromosomes " divide "—or more strictly, perhaps, reproduce themselves —has been the subject of dispute (Darlington, 1935, *b*).

Recently, however, a new method of study has been introduced. Instead of simply comparing the succession of stages in fixed or living cells Kuwada and his collaborators (1933) have treated the metaphase chromosomes before fixation and have in this way been able to imitate the changes the chromosomes undergo during the reconstitution of the resting nuclei. I have had the privilege of seeing their preparations, and their results have been repeated and confirmed in this laboratory (La Cour, 1935; Upcott and Richardson, unpublished).

The preparations of Kuwada and Nakamura (1933, 1934, *a*, *b*) show that each of the four chromatids of the bivalent chromosome at the first metaphase of meiosis in *Tradescantia* consists of a thread compactly coiled in a small spiral, forming a cylinder which is itself coiled in a larger spiral, this being the spiral recognized by many cytologists since the observations of Baranetsky in 1880 (fig. 21). Kuwada (1932), following Fujii (1926) and Shinke (1930), describes the spirals as " primary " and " secondary." But since the order in which they develop is not necessarily the order in which they are revealed to the observer, as Kuwada and Nakamura admit (1933, p. 135), this style is somewhat confusing, and I shall refer to them as the *minor* and *major* spirals, a description about which there can be no misunderstanding. When both the spirals are relaxed by treatment with ammonia vapour, a structure reveals itself which is indistinguishable from that of a resting nucleus except by the absence of nucleoli.

This experiment solves three important problems at once. First, it shows the structure both of the resting nucleus and of the active chromosomes in terms of a permanent element, the chromosome thread. The resting nucleus consists of chromosome threads with their spirals more or less relaxed or uncoiled, the active chromosomes consist of the same threads with their spirals compact or coiled. The experiment shows therefore, secondly, that the thread structure of the chromosomes is maintained from one division to the next. It thus vindicates the theory of permanence and continuity.

And finally the experiment removes the difficulties that have surrounded the question of the time of division of the chromosomes. It has often been supposed that a simple spiral observed at metaphase or later was split. It is now clear that such a split would cut across the minor spiral and must in fact depend on the apparent doubleness of the single cylindrical structure given by the minor spiral.

It is now necessary to determine the dynamic properties of the system

which has been statically revealed to us. We must find out whether the structure of the chromosomes in organisms other than *Tradescantia* is susceptible of the same interpretation at meiosis, whether a similar interpretation can be applied to mitosis, and whether the stages of prophase and telophase give any suggestion of the integration and disintegration of major and minor spirals such as is suggested by Kuwada and Nakamura's experiments.

For this purpose a study of meiosis and mitosis must be made in a group of organisms with large chromosomes. This study must be carried out with a method different from that used in previous studies of the kind. The compatibility of every observation with every other must, therefore, be examined not only in regard to its spatial content, which has always been considered in the past, but also in regard to its mechanical and physiological content, which has been as consistently neglected. To take for granted the empirical validity of observations made on unstable living structures in artificial conditions is always open to suspicion. But when these structures are smaller than the wave-length of light and have had to be coagulated by fixation before they could be seen, we must take advantage of every precaution that inductive-deductive method has to offer us. If we do not go beyond the facts with hypothesis we shall certainly never reach them with observation. The data to which we must chiefly turn are those derived from the prophase of meiosis, where a consideration of the complex evolutions of the chromosomes will often make it possible to say at once that *this* hypothesis will work and that *that* will not.

Preparations of various species of *Fritillaria* made in the course of a survey of the genus (of which I have already given some account—1929, 1930, 1935, *a*) enable me to undertake a preliminary study of this kind. These preparations show the structure of the chromosomes at metaphase, their development at prophase, and dissolution at telophase, and the relationship in these respects between meiosis and mitosis. They are of various kinds. First, there are exceptional smears of pollen mother-cells fixed in medium Flemming solution which, perhaps owing to pressure in smearing, reveal the internal structure of the chromatids and their external relationships. Secondly, there are interphases stained in gentian violet of a quarter strength, showing structure that is obscured by full staining. Thirdly, there are faded preparations which are of value from a different point of view—showing the attachment chromomere still stained, at metaphase of the first meiotic division—but are useless for showing spiral structure. Fourthly, there are preparations of the division in the pollen grain, which shows the first post-meiotic prophase.

Finally there are root-tips fixed in La Cour's (1931) 2 BE fixative. These have been cut at 8 μ for photographing instead of the usual 40 μ.

I am indebted to Mr. La Cour for the preparations and to Mr. H. C. Osterstock for the photography.

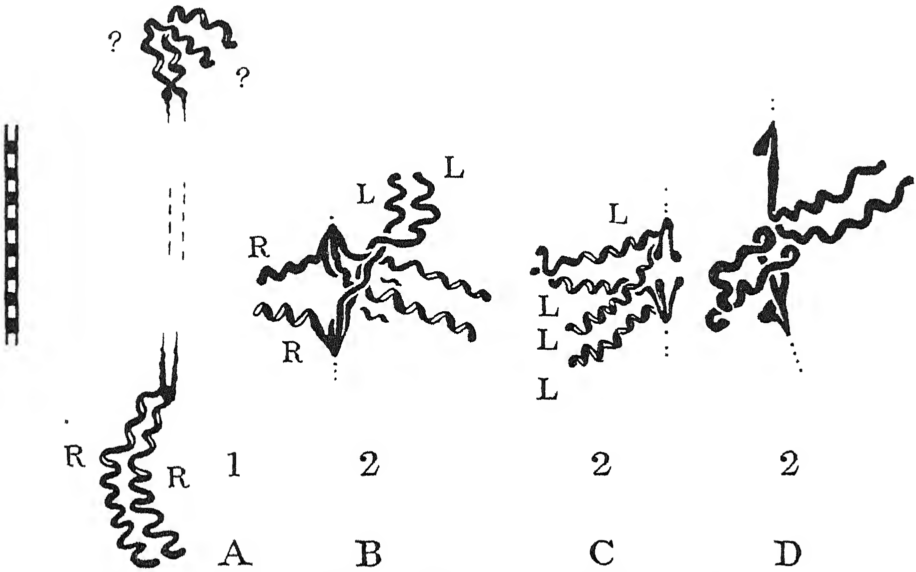


FIG. 1—First metaphase bivalents in diploid *F. latifolia* (same as Plate 2). Direction of coiling given where clear, left-, L, or right-, R, hand screw. The tops of the coils are shown solid. A has a single terminal chiasma in the short arm; B has one chiasma in one arm and two compensating chiasmata in the other; C has a single chiasma in each arm; D has two compensating chiasmata in one arm. The chromosomes are single at the point of the spindle attachment and compensating chiasmata in B give interlocking, while those in D do not, possibly owing to the crossing-over relationships involved. On left, scale of wavelength of Hg line of spectrum. × 3000. Note.—Right and left spirals (corkscrew-wise and anti-corkscrew-wise) are descriptions applicable to a spiral considered in both directions and in both object and microscope image (although not in object and plane mirror image which reverses the direction)

2—First Metaphase of Meiosis

The commonest special type of preparation is that illustrated in fig. 3, and figs. 25 to 34, Plate 2. It shows, first, the paired chromatids of each chromosome widely separated but parallel, except where they change partners with chromatids of the associated chromosome at chiasmata; these chiasmata, whose existence can only be inferred in ordinary preparations from comparison with earlier and later stages, can now be seen

directly at metaphase. Secondly, the preparation shows that the paired chromatids come together at one point in their length, the spindle attachment. This is necessitated by the undivided condition of the attachment chromomere at metaphase, as I have seen it in faded preparations of *F. meleagroides* and elsewhere in which this body has remained stained. Finally, the spiral structure may be seen. Each chromatid appears as a thread wound in a single spiral. This thread occasionally appears double. The direction of the coiling may often be ascertained and appears to be consistent on one side of the spindle attachment.

In other preparations the paired chromatids lie closer together and their spiral coils are more compact. These may be striated if faintly stained or merely corrugated if heavily stained, fig. 3. In either case the

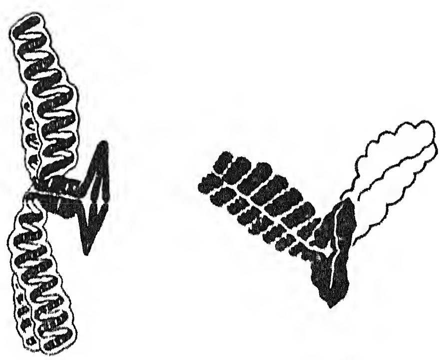


Fig. 2 Fig. 3

Figs. 2 and 3—Lightly stained and heavily stained bivalents of *F. karadaghensis* and *F. latifolia* respectively, showing that the major spiral is made up of a minor spiral. Both have been pressed in smearing. × 3000

doubleness apparent with the other fixation is no longer recognizable. On the other hand, the interpretation of the thread as itself coiled into a minor spiral satisfactorily accounts for these observations and also for the apparent doubleness which is evidently due to the closely coiled and hollow spiral being seen in optical section.

3—Interphase and Second Division of Meiosis

At the first anaphase the major spiral is revealed in one of two ways according to the fixation; it appears as a corrugation on the surface of he darkly stained chromosome, fig. 4, or internally as a contracted spiral coil, fig. 5.

At telophase the nucleus is strongly contracted, but expands immediately into the prophase. Probably no true resting nucleus is formed and

nuclei with no recognizable structure are the result of bad fixation. Nucleoli are developed in some species (*F. latifolia* and the triploids of *F. recurva* and *F. dasyphylla*) but not in others (*F. Eggeri*). I have not been able to determine any associated differences of behaviour. As the nucleus expands the chromosomes appear very much as in the exceptional

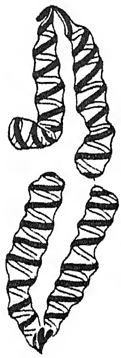


FIG. 4

FIG. 5

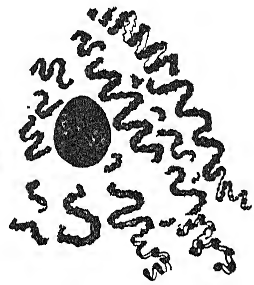


FIG. 6

FIGS. 4 and 5—First anaphase of daughter bivalents in *F. karadaghensis* and *F. latifolia* respectively, indicating major spirals with different types of fixation. × 3000. FIG. 6—First telophase in diploid *F. recurva* showing the opening out of the major spiral to give relic spirals throughout the nucleus and of the minor spiral in the outlying ends of chromosomes. × 3000

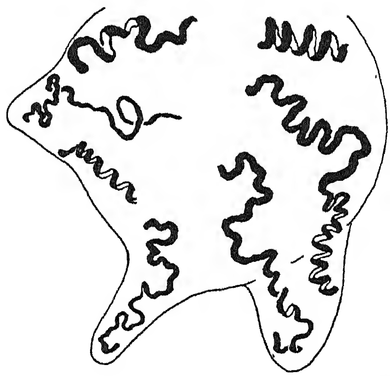


FIG. 7—The same as fig. 8, resulting from pressure. The relic spirals are of different diameters owing to different degrees of uncoiling. × 3000

and treated metaphases, fig. 8 B. Some show merely the regular major spiral, others have indications of the minor spiral, fig. 6. Three changes then occur: first, the mutual adaptations of the expanding spirals distort them into larger loops. Secondly, the major spirals, which may now be called *relic spirals*, uncoil, that is to say they become larger and less numerous. Thirdly, the spiral thread becomes thicker, presumably by

the invisible assumption of internal spirals, the developing major and minor spirals of the new division, figs. 8 C and D.

It seems that the minor spiral does not normally relax during the meiotic interphase and the configurations characteristic of early prophase of mitotic divisions are not seen. But occasionally this spiral is exposed. This is due either to a portion of the chromosome being left protruding from the telophase nucleus, fig. 6, or to the ends of chromosomes being caught and dragged by the knife in smearing, fig. 7. Possibly the failure to relax the minor spiral in interphase is responsible for the chromosomes retaining their special meiotic contraction at the second division in many organisms.

The two chromatids of each second division chromosome, unlike those of a divided mitotic chromosome at prophase, are not derived from

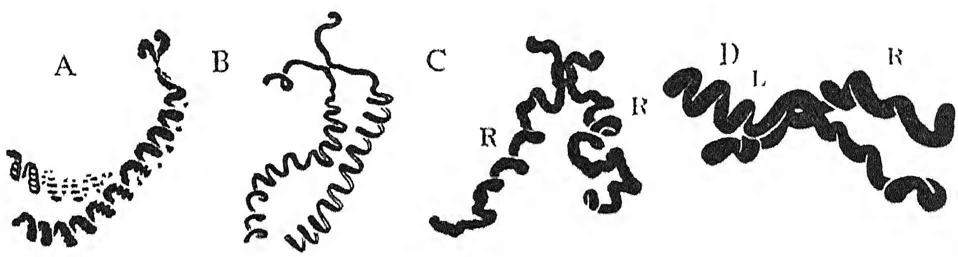


FIG. 8.—Daughter bivalents in the interphase nucleus. A, telophase in *F. gracilis*, B, the same stage with different fixation in triploid *F. dasyphylla*; C, mid-prophase of second division in the same plant; D, later prophase, M bivalent in *F. Eggeri*. × 2000

one chromatid of the previous division with a spiral system in common, but from two chromatids with two separate relic spiral systems which remain separate during interphase. These therefore uncoil separately. Owing to the rapidity of the prophase, as compared with that in ordinary mitosis, the coils are retained until the second metaphase in varying degrees. In consequence the degree of contraction of the metaphase chromosomes as well as their method of association is variable. In some the chromatids lie together throughout their length, in others they come together at only one point, in others again they never come near to one another at any point (apart from the spindle attachment where they are one) before the anaphase division and separation of the attachment chromomeres begin, figs. 9–11.

Another respect in which the behaviour of the spirals is variable is the development of the major spirals of the second division. As a rule it seems that the relic spirals uncoil during prophase and the new major spirals develop inside the chromosome (and invisibly according to the

present technique). Sometimes, however, a closely coiled structure is seen at the second metaphase, in which the direction of coiling can be determined, fig. 9. It seems that in such a case, owing to the rapidity of development, the relic coils of the first division have contributed to the major coils of the second. This possibility is important in relation to what we shall see in mitosis.

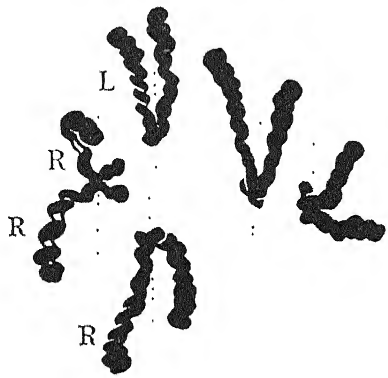


FIG. 9—Five chromosomes of *F. citrina* in process of orientation on the second metaphase plate. The direction of the major spirals is clear in some chromatids. These have perhaps developed directly from relic spirals. × 2000

FIG. 10—Three chromosomes at the beginning of second anaphase in *F. Elwesii* showing separation after different degrees of intimacy of contact in metaphase. × 2000

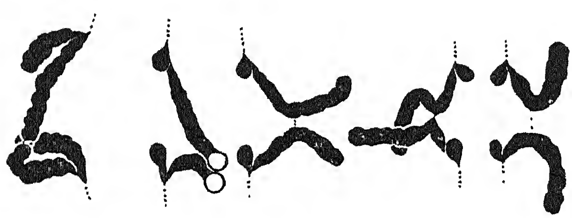


FIG. 11—A later stage illustrating the results of the same differences in earlier association. The first is an M chromosome. × 2000

At an early telophase I have seen the major spirals relaxed in *Fritillaria pudica*. In later stages no exact structure could be made out.

4—Somatic Mitoses

As the chromosomes pass into the resting stage they reveal their spiral structure. First, at early telophase a thick thread of uneven outline appears in a coil of a diameter corresponding more or less to that of the minor spirals at meiosis. Later the thread becomes thinner and of even

outline in a coil of larger diameter. This may be due to the relaxing of a spiral of even smaller diameter, a minimum spiral, which cannot be directly observed. The extension of the minor spiral, whatever its cause, seems to have the effect of distorting the whole thread into a zig-zag, which also assumes an irregular spiral form of larger scope than the other spirals. I shall refer to this as a *super-spiral*. It is occasionally seen in the meiotic interphase, fig. 8*c*. It develops in telophase, fig. 13, although, as we shall see, it does not reach its maximum development until the ensuing prophase. Evidently these changes are parallel with those occurring in the meiotic interphase, with the difference that they go farther as a result of greater extension of the thread.

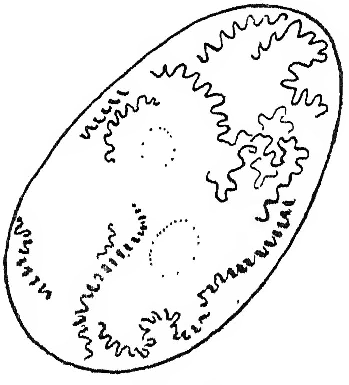


FIG. 12

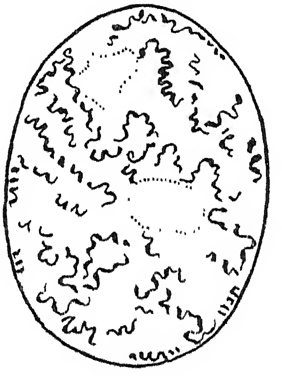


FIG. 13

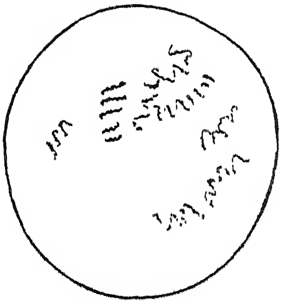
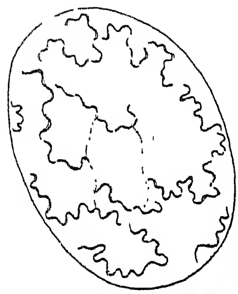


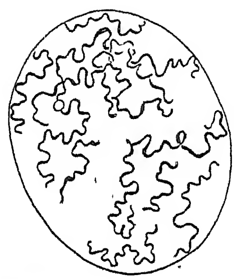
FIG. 13*a*

FIGS. 12 and 13—Telophase in root-tip mitosis. Fig. 12, *F. pluriflora*; expansion of the minor spiral to give larger spiral loops and distortion of the straight direction of the chromatids into super-spirals. Fig. 13, *F. verticillata*, slightly later stage, staining becoming irregular. Fig. 13*a*, latest telophase observable with Flemming fixation. × 2000

Where failure of synchronization has been observed between the parts of one chromosome, nuclei are of critical importance in showing the change of structure during telophase, figs. 12 and 17. Apparently when the spirals are fully expanded and the thread reaches its maximum length and minimum thickness, we have the condition of the complete resting stage in which the thread is no longer strong enough to resist the effects of fixation but breaks up into irregular granules or branches to form the so-called reticulum. The organically permanent structure of the chromosomes is thus temporarily obliterated during the resting stage with Flemming fixation. In the past a maintenance of this structure has been inferred from a comparison of the last stage of the telophase which precedes the resting stage with the earliest prophase which follows it

(Darlington, 1932). This view has been confirmed by Kuwada and Nakamura (1934, *a*, *b*) in direct observations of aceto-carmine fixations. In the intervening period apparently no change takes place in the arrangement of the chromosomes. The name "resting stage" accurately describes its mechanical properties. But as the prophase begins, the large super-spirals, secondary loops or contortions resulting from the redistribution of spiral torsion, become more strongly developed, figs. 14–16, and the straight general arrangement of each chromosome, characteristic of early telophase, fig. 12, is no longer found.*

Fig. 14 

 Fig. 15

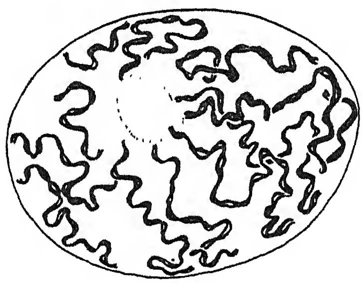


Fig. 16

Figs. 14 to 16—Successive stages of prophase in root tip of *F. pluriflora*. The super-spiral becomes more important while the relic spiral gradually disappears. In the later stages the telophase polarization is seen to have persisted. × 2000

The chromosomes in prophase retain their arrangement with the spindle attachments pointing to the poles of the previous telophase, fig. 11, as observed by Belar (1929). As prophase advances, four changes occur. One of these is internal and its *modus operandi* is not directly visible. This is the contraction in length and expansion in diameter. Presumably the change is due to the assumption of the spirals of the new division corresponding in structure to those revealed in the telophase of the old

* The figure of prophase given by Sharp (1929) has the straight arrangement of coils without any derived super-spirals, characteristic of the early telophase described here. I should interpret it as a telophase.

division. The other changes are external and can be readily followed. They are concerned with the effects of the survival of spirals from the preceding division. The situation has altered during the resting stage in one respect. The chromosomes have divided into pairs of chromatids. These are closely associated and have their relic spirals and super-spirals in common. In early prophase, however, the two chromatids are tightly coiled round one another. The amount of this inter-chromatid coiling, or *relational spiral* as I shall call it, decreases as the prophase advances. The relic spirals and super-spirals also decrease in number, fig. 17.

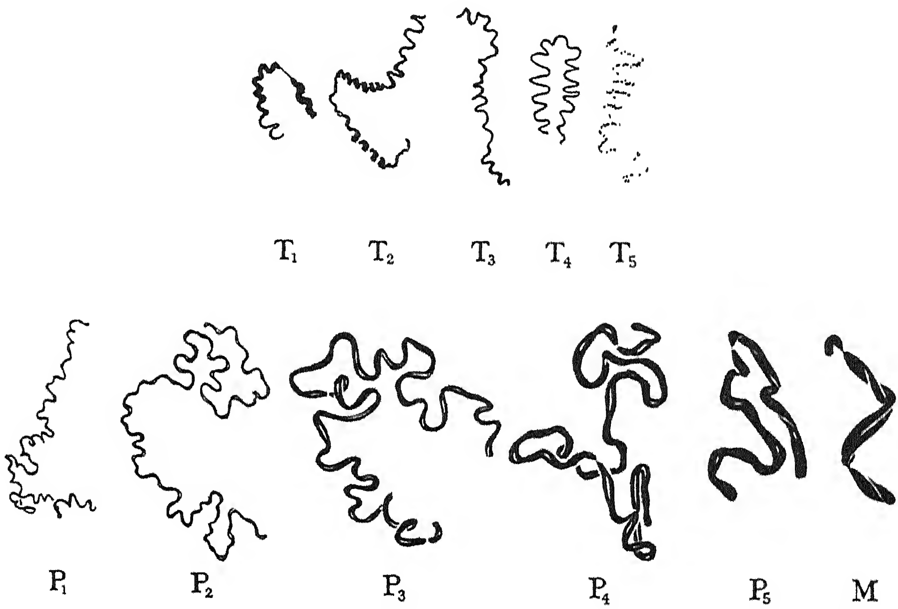


FIG. 17—The successive stages from telophase (T), through prophase (P), to metaphase (M) in a root-tip mitosis of *F. pluriflora*. T1, minor spiral relaxed, loops larger in distal part. T2, slightly later stage. T3, minor spiral expanding, and T4, now uniformly expanded. T5, irregular fixation of chromosome thread when minor spiral is almost completely expanded, beginning of resting stage. P1, P2, and P3, development of super-spirals. P4, P5, whole chromosomes retaining the super-spirals, while the relic spirals disappear, and showing interchromatid coiling. M, super-spirals and chromatid coiling still present. × 2000

By metaphase both have usually disappeared, but the occasional survival of residual coiling of both the interchromatid and joint-chromatid types is responsible for some of the characteristic features of the metaphase chromosomes in *Fritillaria*. I shall return to consider its significance later.

5—Post-Meiotic Prophase

The prophase of the first division in the pollen grain resembles a somatic prophase except that the super-spirals are more clearly developed, owing presumably to the greater stress from the expansion of both major

Fig. 18

Fig. 19

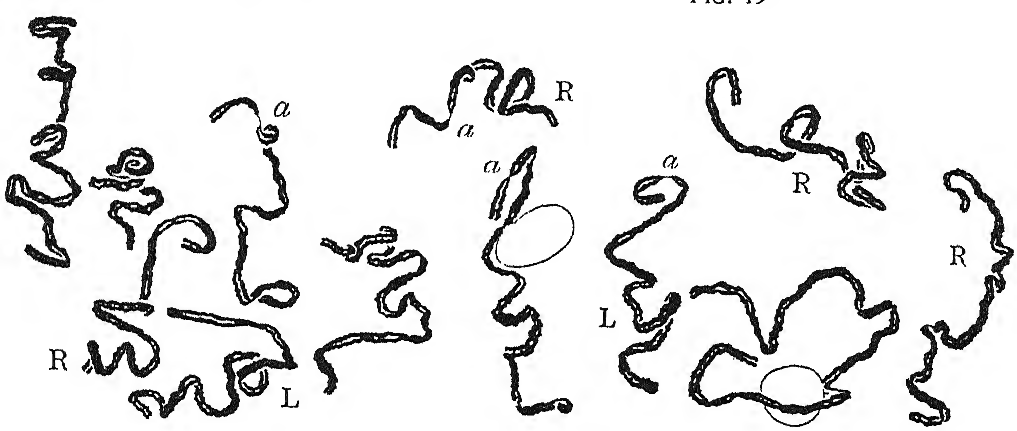


Fig. 20

Figs. 18 to 20—Successive stages of prophase in the pollen grain mitosis (first division after meiosis) in *F. aurea*. In the earliest stage relic spirals and super-spirals occur together; later one or other kind disappears. The direction of both is consistent throughout the chromosomes shown in fig. 18. The chromosomes retain their telophase polarization. M, median chromosome, S, sub-terminal chromosome, *a*, spindle attachments. R, corkscrew-wise super- or relic-spiral (right-hand twist); L, anti-corkscrew-wise (left-hand twist). *r*, right-hand twist of inter-chromatid coiling. Fig. 20, complete set of 12 chromosomes. × 2000

and minor spirals in the preceding telophase, fig. 18. These spirals seem to follow a consistent coil like the major spirals and may therefore be supposed to arise from a redistribution of the same torsion. Variation in the importance and disposition of the relic major spiral and the derived

super-spiral no doubt depend on the relative sizes of the nucleus and the chromosomes.

The relational coiling of the chromatids can be seen to correspond approximately in number to the surviving super-spirals. At a late stage, fig. 19, they have been seen to correspond in direction.

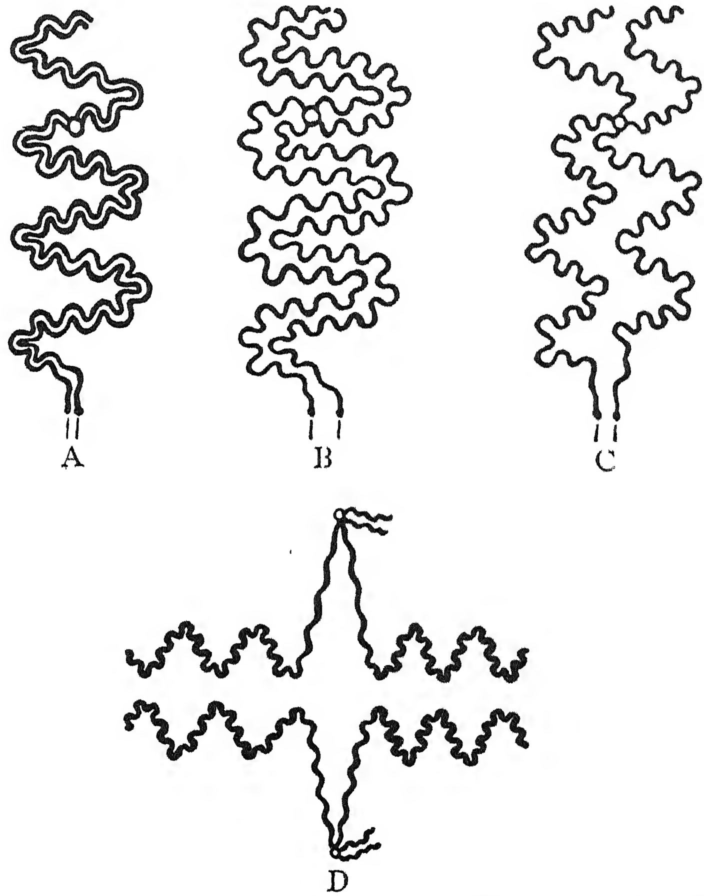


FIG. 21—Diagram of chromatid structure of bivalents at the first metaphase of meiosis showing major and minor spirals of the chromosome thread and spindle attachment chromomeres (circles), and chiasmata. A, B, and C, possible interpretations of one chromosome in *Tradescantia* with a terminal chiasma, all of which may be made in the same preparation (*cf.* La Cour, 1935). A is favoured by Kuwada and Nakamura (1933). C corresponds with that found in *Fritillaria*, D, figs. 3 to 9

6—THE NUCLEAR CYCLE

The present observations agree with those of Kuwada and his collaborators in showing that the chromosomes at the first metaphase in meiosis

are made up of major and minor spirals. The paired chromatids in *Fritillaria* have separate spiral systems, however, united only at the spindle attachment, and not fused spirals as in *Tradescantia*, fig. 21. The difference is perhaps associated with the difference in prophase development which is direct in *Fritillaria* and indirect, passing through a diffuse stage, in *Tradescantia*.

In the following interphase the major spirals are relaxed and become visible just as they do following treatment at metaphase. The second division follows rapidly without any division of the chromatids and the relic major spirals may take part in the formation of the new spirals of the second division. The changes at the meiotic interphase may therefore be said to be mechanically reversible.

With a longer interphase this would perhaps not be true, but variations in this respect are less decisive than those shown in *Gasteria* by Tuan (1931). Here one may suppose after a longer interphase the major spirals of the first division is uncoiled and does not reappear at the second.

The metaphase chromosomes at mitosis are less contracted in length. Their structure is shown by their telophase behaviour to be analogous with that at meiosis except that there is only one demonstrable system of spirals. Upcott (1935) has shown these spirals directly in pollen-tube mitoses of *Tulipa*, about 35 coils in one chromosome. The change at telophase in mitosis, however, consists in a complete relaxation of these spirals. The resting stage follows this relaxation and it is difficult to avoid the conclusion that the resting condition is associated with the relaxation just as in the formation of the artificial resting nucleus by Kuwada's treatment.

The relaxation of the spirals at telophase does not mean their immediate straightening out or uncoiling. A great deal of movement would be necessary for this change and the space is restricted. The uncoiling proceeds very gradually and is far from complete when the resting stage supervenes. The correspondence of the spirals seen at early prophase with those seen at telophase shows that no great change of position has taken place during the resting stage—that it is mechanically what its name implies.

We may now therefore, as a first approximation, define the resting stage as the part of the nuclear cycle in which the spirals of the chromosomes are relaxed. It shares with the diffuse stages, sometimes seen in the prophase of meiosis, an optical homogeneity and an absence of coherent differential staining capacity. No considerable change in the spatial relationships of the permanent parts of the chromosomes occurs

during the resting stage, which therefore presents not a mechanical problem but a chemical one—the problem of the association and dissociation of " accessory " materials, chiefly water, with the " permanent " ones.

During the resting stage, however, the chromosomes have changed in one respect; they have divided into paired chromatids. These chromatids separate at the following anaphase and have separate spiral systems at metaphase. As they contract in length during prophase they must develop these new spirals. It might be expected that the spirals would be the direct successors of the old ones, as possibly happens at the meiotic interphase. But, if they were, the daughter chromatids, which have relic spirals in common, would also have their new spirals in common. That they have not we can see in this way: the relic spirals imply the existence of a compensating relational spiral of chromatids in the opposite direction. A relational spiral can be seen to uncoil during prophase and may even persist until metaphase. The relic spirals are not absorbed by the developing spirals of the new division; they are superimposed upon them. We must suppose, therefore, that they are mechanically independent of them.

The relational spirals should disappear with the relic spirals, because they are reciprocal expressions of the same property. Since, however, they often survive them at metaphase, and since relational spirals can sometimes be seen in the same direction as relic spirals, it seems that such spirals may also develop secondarily owing to the chromatids slipping round one another in such a way as to reduce their relational coiling while the relic coiling is still present. Such an adjustment would yield relational coiling in the same direction as the relic coiling.

Contrary to the generally accepted view, therefore, the prophase of mitosis is not a simple reversal of the telophase. Chemically, no doubt, prophase reverses telophase. Also in regard to the extension and contraction of the chromosome thread this is true. But in regard to the external coiling this is not so: the division of the chromosome and the delay in uncoiling make it impossible. Prophase is a mechanical continuation of telophase uncoiling. We may say that the spiralization is reversible, but its mechanism is irreversible.

7—The Relationships of the Spirals

Comparison of the observations of meiosis and mitosis indicates that the metaphase chromosomes are made up in meiosis of major and minor spirals and in mitosis of minor spirals. In both a third, minimum spiral,

at present beyond the range of vision, may underlie these. This classification is simply morphological. It is possible, on the other hand, that from the development point of view the largest spirals in the two types of division correspond and that in the one division these two spirals are each of larger radius than in the other, but there is no evidence yet to test this. The spiral structure can be shown directly in meiosis, and directly or indirectly in mitosis, where the spirals relax at the telophase. The same structure is probably true of plants and animals generally, but in smaller chromosomes than those of *Fritillaria* the minor spiral as well as any possible minimum spiral will be ultra-microscopic.

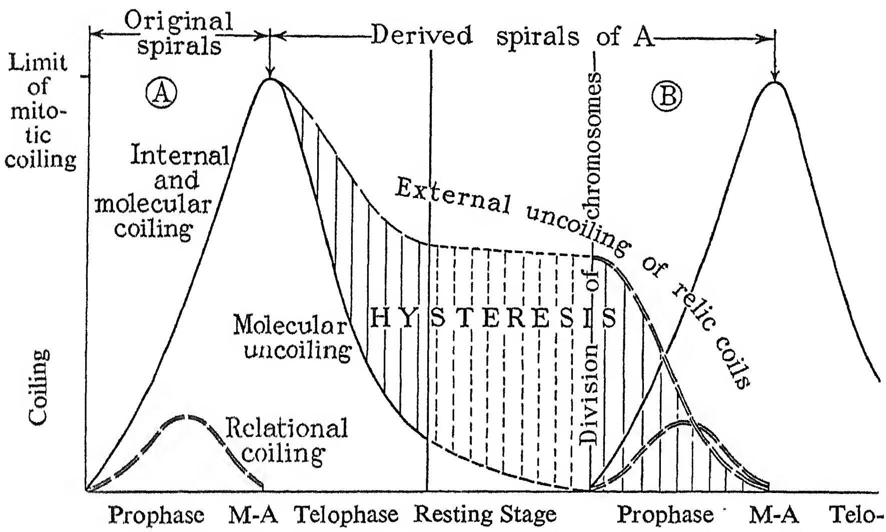


FIG. 22—Diagram showing the spiral behaviour and causation at mitosis and the relation between the spirals seen at succeeding divisions, A. B. The degree of molecular coiling is taken to be the reciprocal of the observed length of the chromosome thread. M-A, metaphase or beginning of anaphase. The arrows represent the division of the attachment chromomere

The failure of the chromosomes to relax their minor spirals during the meiotic interphase where the major spirals relax is no doubt responsible for the retention of the exceptional meiotic contraction at the second division. It suggests also that the major spiral is not developed side by side with the minor spiral but after it, thus reversing the order of their relaxation. The structure of the chromosomes during the prophase of meiosis, to be described later, also supports this view. But many different degrees of contraction at metaphase are found within the same species or within groups of species when we consider the relations of mitosis and meiosis and of meiosis in different races (*e.g.*, in *Matthiola*). This

variation is difficult to understand without a variation in both spirals and suggests a concomitant development of the two. We therefore need direct evidence of the assumption of the spirals in prophase, such as is not yet available.

The relic spirals in the earliest prophase of mitosis have not been related numerically with the minor spirals of the preceding division on account of the difficulty of determining complete chromosomes at an early stage. In mid-prophase they are fewer than the minor spirals of the parent chromatid, which probably number about 25, but at an earlier stage they are smaller and more numerous. They must, therefore, be connected with the minor spirals, while the super-spirals of prophase are connected with the super-spirals or distortion loops produced by mutual accommodation of the relaxing chromosomes at telophase. In the more rapid meiotic interphase the relationship of the relic spirals and the major spirals is clearer, while the super-spirals appear merely as zig-zags arising from the larger folds in the telophase chromosome, Table I.

8—The Molecular Spiral

When a straight thread takes up a helicoid spiral arrangement it becomes shorter from one end to the other. The process of spiral formation during shortening has not been observed, but comparison of the chromosomes before and after shows that the two processes occur together. Shortening without spiral formation is known in molecules of the protein keratin (Astbury, 1933), but there is an indication in another protein, pepsin, of spiral structure (Bernal and Crowfoot, 1934). It seems therefore necessary on cytological grounds, and reasonable on chemical grounds, to take the relationship of shortening and spiralization as the provisional basis of the internal mechanics of the chromosomes.

When a spirally coiled thread is pulled out straight without any relative rotation at the ends, it is found to be internally twisted in the same direction as that of the spiral in which it was coiled. It follows, therefore, that the assumption of a spiral by a chromosome thread may take place under one of two conditions. Either the spiral arises in the chromosome by rotation of its ends, and without any internal spiral readjustment or twisting, the change presumably being imposed by an external force which is resisted by the internal cohesion of the chromosome thread; or it arises by an internal twisting of the thread in the opposite direction to the spiral assumed and without any rotation of the ends.

The first possibility can be excluded by three chief considerations. First, the contraction of mitotic ring-chromosomes (as in *Crepis*, Navashin

TABLE I—ANALYSIS OF SPIRAL STRUCTURE

Relationship	Structure	Type in order of increasing diameter	Duration: Mitosis (P M-A T)	Duration: Meiosis (P M-A T)
I Internal compensating spiral (1)	Single chromatid (1-4)	A—*Original spirals* (in metaphase of the mitosis of origin)—		
		1 Molecular spiral (R)	——————→	——————→
		2 Minor spiral (L)	——————→	× ————→
II Individual spirals (2-4A) in opposite direction to (1)		3 Major spiral (L)	× × × × ×	× ————→
	Paired chromatids (3A-5)	B—*Derived or transferred spirals*—		
		4 Super-spiral (by transfer from 2 and 3 (L))	× × × × →	× × × × →
	Paired chromosome (6, 6A)	5 Relic spiral from 2 or 3 (L)	——→ × × ×	——→ × × ×
		4A Super-spiral from 4 (L)	————→ × ×	——→ × × ×
III Relational spirals (5, 6)		6 Inter-chromatid spiral (R) (opposite to II)	————→ × ×	× → × × ×
		7 Inter-chromosome spiral (L)	× × × × ×	× → × × ×
		7A Transferred chromosome spiral (L)	× × × × ×	× → × × ×

1930) would be rendered impossible. Secondly, long chromosomes would contract and relax more slowly than short ones, whereas the contraction of chromosomes of widely different lengths is often found to be uniform. Thirdly, rotation is inconsistent with the observations of paired chromosomes with interstitial chiasmata at diplotene.

We must, therefore, conclude that the spiralization is associated with a compensating internal twist due to a rearrangement of the constituent particles, a rearrangement either between molecules or within molecules. I shall refer to this twist as the *molecular spiral*. The degree of coiling of the molecular spiral may be taken to be the reciprocal of the length of the chromosome thread. Deriving its value in this direct way, I shall treat it as an empirical datum, fig. 22.

Since the molecular spiral must determine both the major and minor spirals, these must be in the same direction, and opposite to the twist that conditions or determines them.

Further, since the relaxation of spirals can be produced by changing the p_H of the medium according to Kuwada's method, the molecular spiral is presumably itself conditioned by change in the surface charge, or double electric layer, on the chromosomes, which I have supposed to change during prophase and to be responsible for the movements of the chromosomes, such as terminalization, that I have previously described (Darlington, 1932). We can therefore see for the first time a relationship between the internal and external mechanics of the chromosomes. The molecular patterns that would determine these changes have been considered by Wrinch (1934).

But the deductions from the hypothesis of a compensating molecular spiral that immediately concern us are those that affect the observed uncoiling of the major and minor spirals in telophase and prophase. If the chromosome were a mechanically isolated system, if indeed it could be considered *in vacuo*, a change in the molecular spiral would be immediately reflected in the visible spirals conditioned by it. In other words, the spirals would disappear as the chromosome increased in length and would reappear as it contracted. Prophase and telophase would be reciprocal. This clearly cannot be so. A chromosome is packed with its neighbours inside the nucleus. It is held in a viscous medium within a restricted space in which it is lying in an equilibrium depending upon the mutual repulsions between itself and all the other chromosomes.

These conditions will not obstruct the contraction of the chromosome. It can contract by spiralization without meeting any considerable resistance; there should be a close correlation between the assumption of the spiral and the molecular changes determining it. When the molecular spiral uncoils, on the other hand, the uncoiling of the chromosome will meet with resistance from its neighbours. Only gradually can it be expected to adjust its external form to its internal stresses.

These *a priori* conclusions are verified by the present observations of the gradual and delayed uncoiling of the relic spirals, an uncoiling which continues long after the chromosome thread has reached its maximum length and minimum thickness, and while it is actually shortening and developing the spirals of the new division. We see that the external uncoiling does not correspond in the nuclear cycle with the lengthening which is determined by the internal uncoiling of the molecular spiral. The fewest spirals are not found when the chromosome thread is longest and thinnest. In short, during coiling the molecular spiral and induced spirals correspond; during uncoiling the induced spirals are delayed. The relic spirals of the prophase represent a mechanical lag, an hysteresis.

The chromosomes thus pass two cycles in the course of a nuclear division, one in regard to length and degree of internal coiling, the other in regard to the degree of external coiling. In ordinary mitosis, the length reaches a maximum in the resting stage and a minimum at metaphase or anaphase. The degree of coiling is at a maximum at one metaphase and does not reach a minimum until the ensuing metaphase, two systems of coiling, the new internal and relic external coils, coexisting during the prophase.

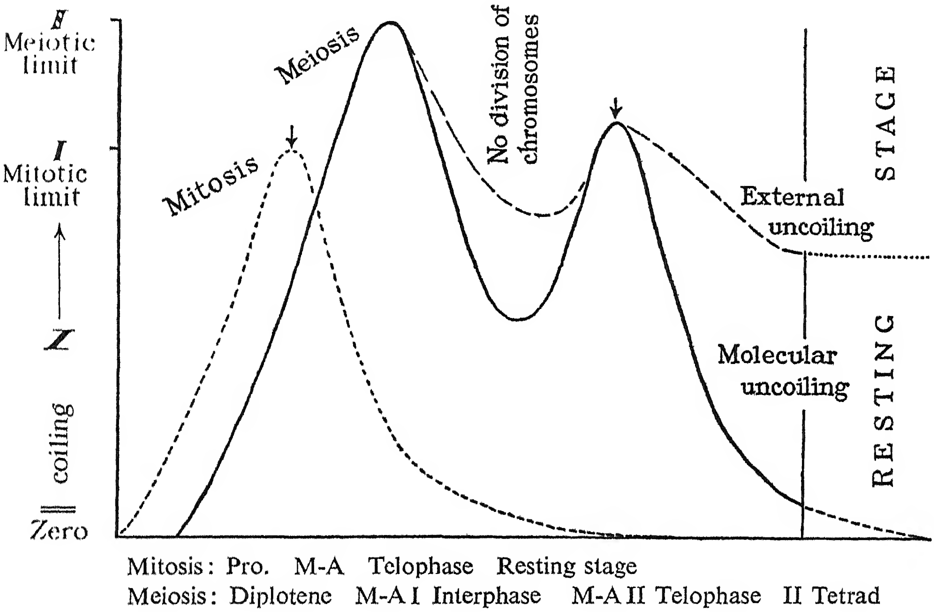


FIG. 23—Diagram showing relation between spirals seen at meiosis and those at mitosis, fig. 22, and the difference between interphase and an ordinary resting stage

The hypothesis of a molecular spiral enables me to express the relationships of these empirically observed changes quantitatively for analytical and inductive purposes. I have accordingly used it to represent graphically the formation of spirals at mitosis and meiosis, figs. 22 and 23. The degree of molecular coiling is taken to vary in inverse proportion to the *length* of the chromosome thread. The degree of external coiling is measured by the *frequency of coils* observed in a given length of chromosome, apart, that is, from the coiling which may be developing internally. The maxima of the two agree, the minima do not. The maxima lie at metaphase or anaphase (M–A) in both cases. In mitosis the minimum external coiling is reached at the end of the metaphase of the following

division. The minimum molecular coiling is reached during the resting stage. The exact time is probably the end of the resting stage, since in meiosis with a precocious prophase the minimum is not reached until the middle of prophase, the end of the pachytene stage. In both meiosis and mitosis it possibly therefore coincides with the time of division of the chromosomes. The detailed evidence for this view will be given in the next study.

In the meiotic diagram I have shown the extreme opposite from the behaviour in mitosis. With a short interphase the chromosomes are represented as absorbing the relic spirals of the first division to form the major spirals of the second division. How far this is so in *Fritillaria* I do not know, since the distinction between relic and new major spirals is, as I have pointed out, not always clear. In the mitotic prophase it must always be clear, since the one involves paired chromatids, the other only single chromatids. In the second meiotic prophase, on the other hand, the same structures are involved in both, owing to failure of division during the interphase.

The question of the causation of the spirals is wrapped up with another question on which direct evidence can be brought to bear. I have not observed changes in the direction of coiling of the arms of chromosomes (on either side of the spindle attachment). This uniformity in each arm, found also by Nebel (1932, *a*, *b*), implies that the direction of coiling is subject to unitary control. A contrary observation of changes of direction in particular arms would imply that the direction of coiling was a genetic property of particular segments, *i.e.*, was both hereditary and specific to these segments. This property would, therefore, be unaffected by structural changes. Such changes occurring at random in the course of evolution, as they seem to do, would then yield a random assortment of parts of opposite coiling types. The characteristic coiling types of chromosome arms that we now recognize would long since have disappeared.

There are also other reasons for doubting the existence of changes of direction in coiling. They would show by the unequal yielding of the parts concerned wherever, as at anaphase, the chromosome was subjected to exceptional stress. Changes of direction might be supposed to be responsible for secondary constrictions; but we already know that such constrictions are indeed the genetic property of the segment concerned (McClintock, 1934), and are associated with the second genetic property of nucleolus formation (Heitz, 1931). Finally, the assumption of changes of direction seems to be inconsistent with the data I shall describe later on relational coiling and crossing-over.

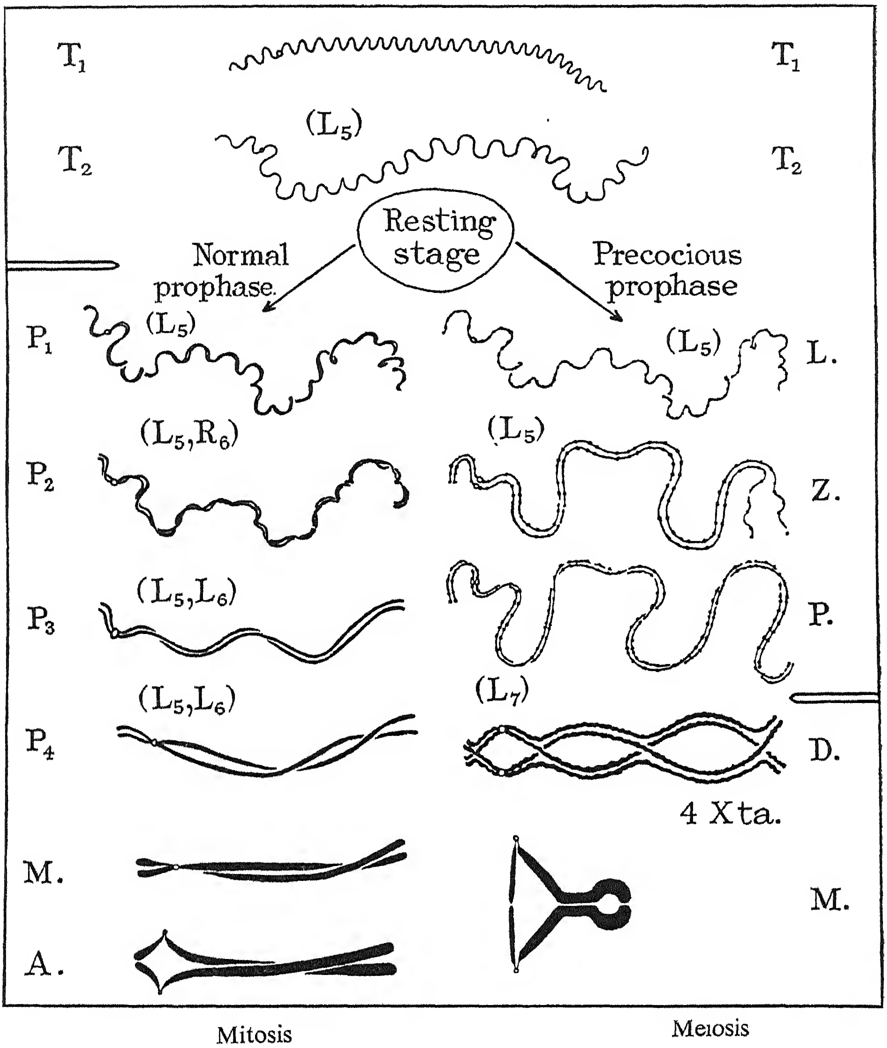


FIG. 24—Diagram to show the history of spiral structures in the nuclear cycle, mitosis and meiosis, in relation to the division, represented by double lines in the side columns, and crossing-over of chromosomes, represented by chiasmata, Xta. With normal prophase the chromosomes divide at the end of the resting stage, with the precocious prophase at the end of pachytene; in both cases when the chromosomes are at their longest. T, telophase; P_1 to P_4, prophase; M, metaphase; A, anaphase; L, leptotene; Z, zygotene; P, pachytene; D, diakinesis. (R) and (L), directions of individual and relational coiling of chromatids at mitosis and chromosomes at meiosis numbered according to the seven categories of Table I. The chromatid coiling at P_4 is opposite to that at P_2 and therefore not derived from it. A circle represents the spindle attachment chromomere which divides at the end of metaphase in mitosis and of the second metaphase in meiosis

I arrive in this way at two conclusions that at first sight seem irreconcilable: the determination of the spiral by the local molecular constitution of the thread and the unitary control of its direction in each arm of a chromosome. The contradiction is perhaps highly significant. It can be resolved by assuming that while the coiling itself is locally determined, its direction is determined by some structure in respect of which the arms of chromosomes may be taken as units, *e.g.*, the spindle attachment or a hypothetical pellicle (Darlington, 1932, pp. 290-2).

9—Summary

Special kinds of fixation show the meiotic bivalent of *Fritillaria* with its four chromatids all separate except at the spindle attachment, which is single for each pair of chromatids, as has been predicted on the simplest assumptions from the precocity theory and from direct mechanical considerations, fig. 1; and figs. 25–27, Plate 2.

Under these conditions the chiasmata can also be directly observed and each chromatid is seen to consist of a thick thread wound in a spiral of from eight to fifteen coils. The variation in number and diameter of this *major spiral* in different species is associated with variation in the degree of linear contraction.

The thick thread shown in this way appears with different methods of fixing to consist of a *minor spiral* of about 80 coils. These observations agree with the experimental results of Kuwada on *Tradescantia*, except that the chromatids are coiled separately and not jointly.

In the first meiotic telophase the major spiral relaxes to form a *relic spiral*. This expands its loops and throws the chromatid into larger *super-spirals*. The minor spiral is then visible as a cross-striation in the thread of the relic major coil, but it does not relax.

In the ensuing prophase the chromosomes contract and the relic spirals and super-spirals gradually disappear.

The spiral structure of the chromosomes at the second metaphase is similar to that at the first. The association of the chromatids is often incomplete owing to the rapidity of the division.

The changes occurring during the resting stage between somatic mitoses show the dissolution of minor spirals by relaxation and uncoiling in telophase and by continued uncoiling during contraction in prophase. Different stages are seen in the same chromosome at telophase owing to lack of synchronization.

The chromosome is coiling new spirals *internally* at the same time that

it is independently uncoiling the old ones *externally*. Prophase is therefore in this respect not a reversal but a continuation of telophase.

At the first post-meiotic prophase, in the pollen grain, the relic spirals and the super-spirals similarly disappear during contraction. They are consistent in direction in particular chromosome arms.

The uncoiling of the old spirals after division of the chromosome in mitosis is accompanied by the uncoiling of a compensating *relational spiral* of the daughter chromatids. Both uncoilings are sometimes incomplete in the long chromosomes of *Fritillaria* at metaphase. A process of uncoiling leads after pairing in meiosis to a relational coiling of chromosomes which will be described later.

Both the major and minor spirals may be interpreted from optical sections as double non-spiral threads, and many workers have therefore concluded that the chromosome thread is divided in the metaphase chromatid or telophase daughter-chromosome. This view is inconsistent with (i) the relative dimensions of the major coil at metaphase and the early prophase chromosome, (ii) the observation of the minor spiral, (iii) the results of experimental uncoiling, (iv) the developmental evidence given here, (v) the direct leptotene observations, (vi) the genetic data from X-rayed Drosophila sperm, and (vii) the cytogenetic data from X-rayed tissue of higher plants (*cf.* Darlington, 1935, *b*).

The leptotene observations have in the past conflicted with those of other stages because it is at leptotene alone that the observed chromosomes have no internal spiral. The beginning of the formation of spirals seems to coincide with the division of the chromosome at the end of the pachytene stage in meiosis and at the end of the resting stage in mitosis.

The study of the internal mechanics of the chromosomes must rest on the following general assumptions, *inter alia* :—

(i) The nuclear cycle depends on a reversible change in the arrangement of permanent chromosome threads from one internally stable phase, the resting nucleus, to another, the metaphase chromosomes. In the first the internal condition of the threads acts to straighten them, in the second to determine an arrangement in a single spiral, the minor spiral at mitosis, or a double spiral, the minor and major spirals at meiosis.

(ii) This property of the formation of spirals is directly determined by a molecular property of the thread which leads it to coil in an internal spiral in the opposite direction to the minor and major spirals. The direction of this *molecular spiral* is subject to unitary control in each arm of a chromosome.

(iii) The arrangement of the thread adjusts itself immediately to the change determining the formation of spirals, but not to the opposite

change determining uncoiling on account of the restricted space of the nucleus in the telophase, resting stage, and prophase, the repulsions between the chromosomes, and the viscosity of the nuclear sap. Relic spiral arrangement therefore persists through the resting stage until the following metaphase. Its gradual uncoiling is mechanically independent of the developing spirals of the new division in mitosis, and its persistence varies with the situation of individual chromosomes. If it were not independent the daughter chromatids would be jointly coiled. There is an *hysteresis* in the adjustment of the external form of the chromosome to its internal stresses. The prophase is therefore not simply a reversal of the telophase, but partly a continuation of it.

(iv) The uncoiling of spirals after the chromosomes have divided in the resting stage of mitosis and after they have paired in the prophase of meiosis leads to the disappearance of relational coiling of chromatids in mitosis and the development of relational coiling of chromatids and chromosomes in meiosis.

Description of Plate 2

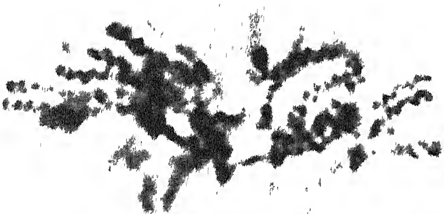
Figs. 25–29—Photographs of two first metaphase plates of diploid *Fritillaria latifolia* taken in side view. Bivalents of the one in figs. 25–27 are drawn in fig. 1. Note the separate coiling of the paired chromatids, the chiasmata, and the singleness of the arrow-head-like spindle attachments. × 2000.

Figs. 30–34—Mitosis in the root-tip of *F. verticillata*. Figs. 30 and 31: telophase showing the straight general direction of the chromosomes which have opened out their minor spirals; the same nucleus at two levels. Figs. 32 and 33: prophase showing relic and super-spirals. Fig. 34: later prophase with super-spirals flattened on the surface of the nucleus and relic spirals chiefly as transverse corrugations. × 2000.

References

Astbury, W. T. (1933). "Fundamentals of Fibre Structure," Oxford.
Belar, K. (1929). 'Z. Zellforsch. mikr. Anat.,' vol. **10**, p. 73.
Bernal, J. D., and Crowfoot, D. (1934). 'Nature,' vol. **133**, p. 794.
Darlington, C. D. (1929). 'J. Genetics,' vol. **21**, p. 207.
—— (1930). 'Cytologia,' vol. **2**, p. 37.
—— (1932). "Recent Advances in Cytology," London.
—— (1935, *a*). 'J. Exp. Biol.' (*in the press*).
—— (1935, *b*). 'Ann. Bot.' (*in the press*).
Fujii (1926). 'Rep. Jap. Ass. Adv. Sci.,' vol. 2.
Heitz, E. (1931). 'Planta,' vol. **12**, p. 774.
Kuwada, Y. (1932). 'Bot. Mag., Tokyo,' vol. **46**, p. 307.
Kuwada, Y., and Nakamura, T. (1933). 'Mem. Coll. Sci. Kyoto Imp. Univ.,' vol. **9**, p. 129.

25

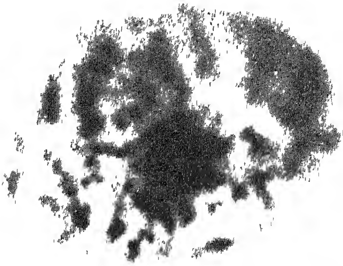


26

27

28

29

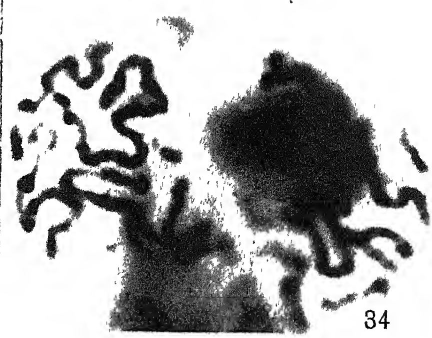


30

31

32

33



34

Kuwada, Y., and Nakamura, T. (1934, *a*). 'Cytologia,' vol. **5**, p. 244.
—— (1934, *b*). 'Mem. Coll. Sci. Kyoto Imp. Univ.,' vol. **9**, p. 343.
La Cour, L. (1931). 'J. Roy. Micr. Soc.,' vol. **51**, p. 119.
—— (1935). "Stain Technology." (*In the press.*)
McClintock, B. (1934). 'Z. Zellforsch. mikr. Anat.,' vol. **21**, p. 294.
Navashin, M. (1930). 'Univ. Calif. Publ. Agr. Sci.,' vol. **6**, p. 95.
Nebel, R. (1932, *a*). 'Z. Zellforsch. mikr. Anat.,' vol. **16**, p. 251.
—— (1932, *b*). 'Z. Zellforsch. mikr. Anat.,' vol. **16**, p. 285.
—— (1933). 'Cytologia,' vol. **5**, p. 1.
Sharp, L. W. (1929). 'Bot. Gaz.,' vol. **88**, p. 349.
Shinke, N. (1930). 'Mem. Coll. Sci. Kyoto,' vol. **5**, p. 239.
—— (1934). 'Mem. Coll. Sci. Kyoto Imp. Univ.,' vol. **9**, p. 267.
Upcott, M. B. (1935). 'Nature.' (*In the press.*)
Tuan, H. C. (1931). 'Bot. Gaz.,' vol. **92**, p. 45.
Wrinch, D. M. (1934). 'Nature,' vol. **134**, p. 978.

576 . 354 . 46

The Internal Mechanics of the Chromosomes
II—Prophase Pairing at Meiosis in *Fritillaria*

By C. D. Darlington, John Innes Horticultural Institution, London

(*Communicated by Sir Daniel Hall, F.R.S.—Received February* 26, 1935)

[Plate 3]

1—Introduction

In the previous study I have described the changes in internal structure that the chromosomes undergo at mitosis and during the later stages of meiosis in *Fritillaria*. I have arrived at certain conclusions as to the relationships of these changes with the nuclear cycle.

The most important of these are, first, that the spiral arrangement of the chromosomes is determined by a compensating molecular change; secondly, that there is a lag in the adjustment of the external form of the chromosome to its internal molecular stresses; and thirdly that paired chromosomes and chromatids will be caused by this adjustment to develop special spiral relationships which I have called relational coiling.

The next task is to find out how far these conclusions are supported by the study of the prophase of meiosis in which the series of changes is

intricate enough and variable enough to provide a test of any general hypothesis of chromosome mechanics that may be put forward.

For this purpose *Fritillaria* is peculiarly useful because its species vary in their prophase behaviour in essential respects. Some species have their chiasmata evenly distributed along the whole chromosome (Darlington, 1930), while others, in fact 15 out of the 25 species that I have studied, have their chiasmata more or less strictly localized in the neighbourhood of the spindle attachment, *i.e.*, in what are called the proximal parts of the chromosomes (Newton and Darlington, 1930). I have pointed out (1932, *a*, p. 98) that in a species with localization, *F. Meleagris*, " small unpaired loops are constantly found at pachytene " and I have suggested (p. 348) that " the distal parts of all the chromosomes are precocious in *Mecostethus* and this is probably responsible for the localization of pachytene pairing and chiasma formation here, in *Fritillaria* and elsewhere." A precocious division of the distal parts of the chromosomes into pairs would prevent the homologous chromosomes associating, since I assume that only pairs of threads attract one another. Huskins and Smith (1934) in a recent study have given an account of pairing in *F. Meleagris* which purports to support this hypothesis.

My present object is therefore to elucidate the particular situation in *Fritillaria* as well as to find out its general bearings on the internal mechanics of the chromosomes.

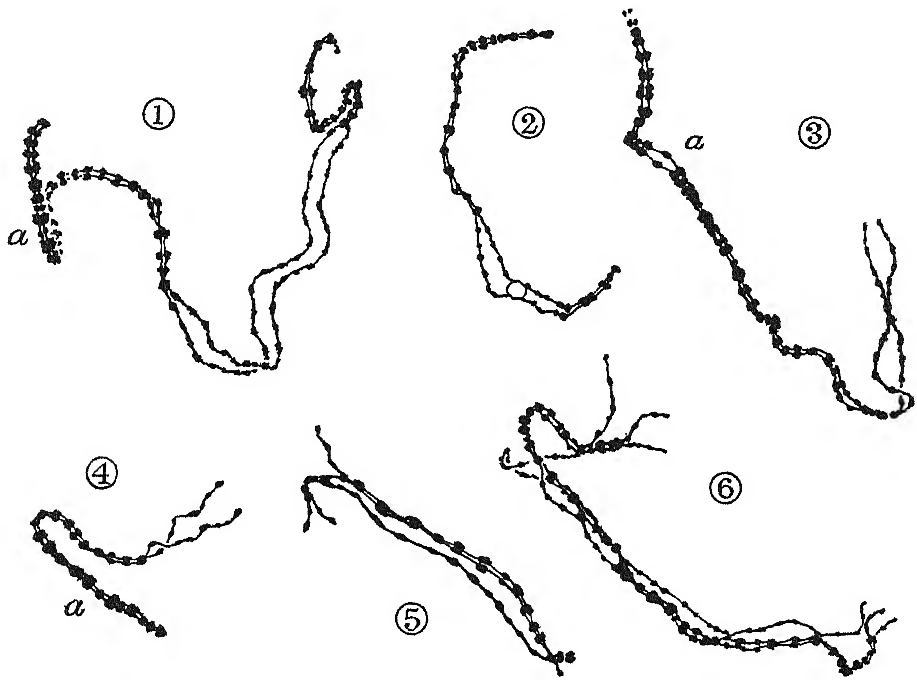
The technique that has been used is referred to in the previous paper. The material that has been studied will be described and classified in a later article. (Darlington, 1935.)

2—The Process of Pairing

In their early prophase behaviour the species with and without localization are indistinguishable, and the same description will apply to all of them.

In the earliest prophase, the leptotene stage, the chromosomes are fine single threads of chromosomes. They can be followed in the plane of focus, but, as in other plants, owing to their fineness and their contortion in relic spirals they are impossible to follow for any great distance and their proximal and distal parts cannot be distinguished. They are presumably lying in the positions in which they were left at their preceding telophase. Later, fig. 24, the spindle attachments lie chiefly to one side of the nucleus, a condition of polarization that is presumably derived without change from the preceding telophase arrangement.

The homologous chromosomes which have been lying parallel, pair side by side to give the double " pachytene " thread, figs. 1-6. As pairing proceeds the chromosomes can be followed for considerable distances. They then show the *Agapanthus* phenomenon of differential condensation (Darlington, 1933). The thread in the neighbourhood of the spindle attachment is more condensed than in the distal regions. The chromomeres are larger and hence appear closer together. The difference is less extreme than in *Agapanthus* and it disappears early in diplotene,



FIGS. 1–6—Zygotene, 1-3 in diploid species, 4-6 in triploid *F. pudica*; showing that the pairing threads lie parallel and that pairing begins near the spindle attachment chromomere (*a*). Fig. 1, *F. imperialis*; fig. 2, *F. pontica* with localized pairing (note non-pairing near small nucleolus); fig. 3, *F. Eggeri*. × 2800

but it is constantly present at pachytene. Differential condensation is shown equally in species with no localization of chiasmata, such as *F. imperialis*, *F. libanotica*, *F. pallidiflora*, *F. Eggeri*, and *F. pudica* (a triploid), in others with intermediate localization such as *F. verticillata*, *F. obliqua*, and *F. oranensis*, and in others again with extreme localization such as *F. acmopetala*, *F. pontica*, and *F. Meleagris*. Probably in some degree differential condensation as between parts of long chromosomes is very general in the higher plants, although it has only been noticed in *Agapanthus*

and *Kniphofia*. In the Orthoptera and Mammalia it seems to be represented by differences between chromosomes of different lengths (Darlington, 1932, *b*).

This differential condensation often enables one, as in *Agapanthus*, to pick out the attachment chromomeres, which appear to be spherical bodies about 0·25 μ in diameter (*a*, in figs. 8, 20, 23, etc.) or approximately the same size as I find them at metaphase in *F. meleagroides* (1935), and at pachytene in *Agapanthus*. They are usually, but not always, closely paired. They are separated by thin threads nearly 1 μ long from the main bodies of the chromosome on either side of them, and, lying as they do in the most condensed part, they stand out clearly from the rest of the chromosomes. Sometimes the angle of the thread conceals them and sometimes there may seem to be two or three separate chromomeres in the gap between the condensed parts of the chromosomes. I found this abnormality in *Agapanthus*, but I do not know whether it is significant. The attachment chromomeres lose their special staining capacity at the beginning of diplotene. They vanish, but their positions continue to be marked by non-staining gaps in the chromosome, figs. 24-28.

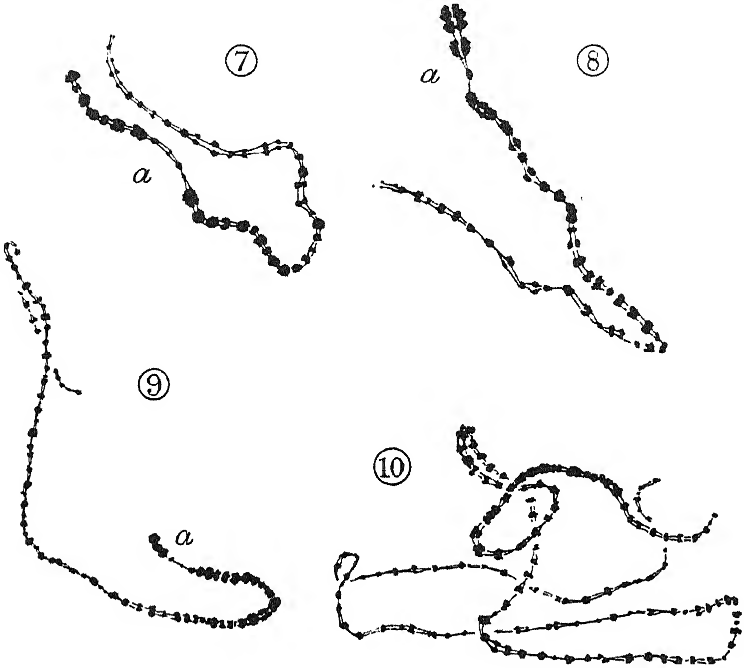
The importance of the attachment chromomere for the present study is that it enables one to identify at once the proximal part of any chromosome and see that it is this part which pairs first as well as condenses first. This also is equally true of species with and without localization, figs. 1, 3, 4, 11, 12, 16.

It seems probable that the earlier condensation of the proximal parts of the chromosomes is not an independent property of these parts but depends on their earlier pairing, since paired parts in general seem more condensed than the unpaired parts adjoining them, in *Fritillaria* as well as in *Tulipa* and elsewhere.

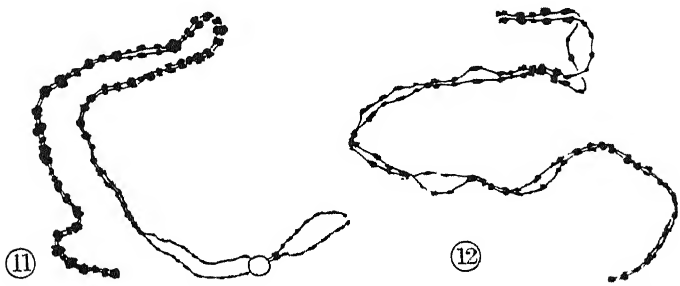
Let us now consider the later course of events. The behaviour of species with localization will be more readily understood if we first take those with a normal distribution of chiasmata. It has always been supposed that pairing in such species was complete. An occasional unpaired segment would be taken as indicating that the nucleus was still in an active zygotene condition. Yet I have rarely found a nucleus with complete pairing, and it can be shown that in the majority of nuclei pairing is never complete by the fact that short distal segments may be seen still unpaired at a time when the diplotene separation has already begun in the proximal segments, fig. 25.

This failure of pairing is found to be very common in the parts of the chromosomes near the nucleolus, figs. 11, 13, 14, 15, and 30. Two stages

may be recognized. First the homologous chromosomes are seen lying more or less parallel in these parts, fig. 13. Later they have become coiled round one another, fig. 14. This relational coiling, since it is the sixth



FIGS. 7–10—Pachytene, showing condensation in the proximal parts of the chromosomes. Fig. 7, *F. Eggeri*; fig. 8, *F. imperialis* var. " Maxima Red "; figs. 9 and 10, *F. verticillata*, a species with semi-localized pairing; distal regions not shown except in fig. 10 where pairing is incomplete. × 2800



FIGS. 11 and 12—Early pachytene in proximal and distal parts of paired chromosomes in *F. obliqua*, with semi-localized pairing. Note parallel arrangement of unpaired threads. × 2800

type of spiral relationship, I have indicated as R_6 or L_6. It is equally found between other unpaired parts in all species at what is presumably late pachytene and at diplotene. It is consistent in direction in each arm

of a chromosome and is also consistent apparently with the relic coiling R_2 and L_2, of which traces may still be seen at the beginning of diplotene, figs. 24 and 26.

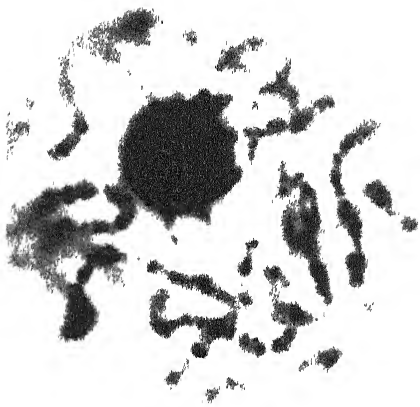
The triploid *F. pudica* shows relational coiling in a different way. The third unpaired chromosome, which at an early stage, when pairing is incomplete, figs. 4-6, can be seen lying parallel with its mates, at a later stage becomes coiled round them, figs. 18-19. The paired as well as the unpaired parts must therefore have the property of coiling, although its consistency cannot be directly demonstrated in the paired parts of the diploid.

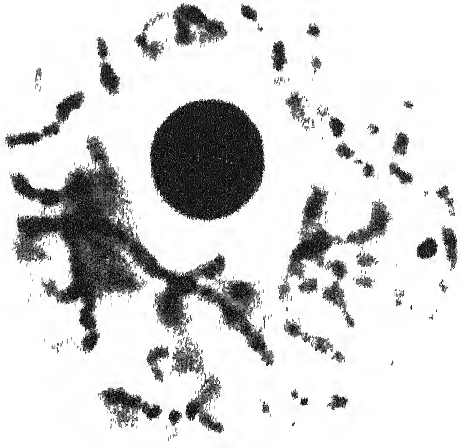
It is important to notice that relational coiling develops only in intercalary unpaired regions, figs. 16, 18, 23. Relational coiling in a particular segment depends on the ends of that segment being associated. If they are free no coiling arises. The coiling therefore expresses a state of stress.

3—The Localization of Pairing

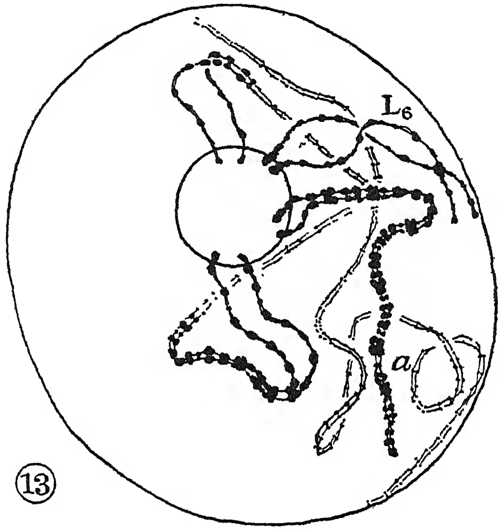
With species having partial and complete localization, pairing begins and precocious condensation develops in the proximal regions, just as with the normal species. But pairing never approaches completion. In certain nuclei chromosomes may be seen with a degree of relational coiling in intercalary unpaired segments that can only have developed during a prolonged pachytene and with a great part of their distal length still unpaired, figs. 22 and 23. The condition in these late pachytene nuclei is precisely the same as that in a zygotene nucleus of a species with normal pairing; association is complete proximally but intermittent or absent distally. Space will not allow me to illustrate the whole of such a late pachytene nucleus, but I estimate that about half the length of the threads is paired and half unpaired in species with extreme localization, while about three-quarters is paired in the intermediate species.

The incomplete pairing in these species, as in the non-localized ones, has two important characteristics. First, it is intermittent, figs. 8, 9, 22, 23, etc. Unpaired segments lie between paired segments. These are the intercalary segments I shall frequently have to refer to in the next article. Secondly it is variable in extent amongst the chromosomes and from nucleus to nucleus. This variability is probably greatest in the species with an intermediate degree of localization and it is perhaps related to the variation in the degree of their metaphase contraction. It is certainly related to the chance variation in the spatial relationships of the pairing chromosomes in the zygotene nucleus.

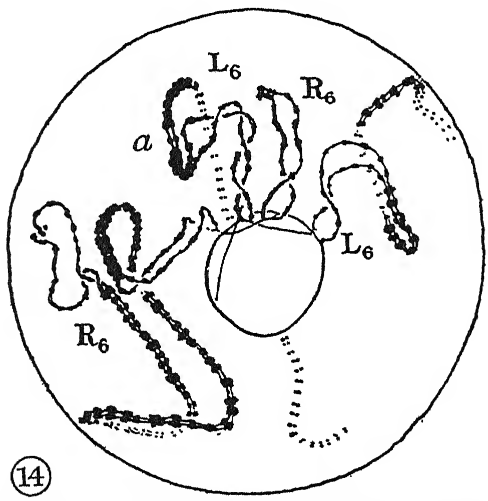




In the later coiled stages of pachytene the larger chromomeres in the unpaired threads can sometimes be seen to be split, although they are even smaller than the paired chromomeres near them. They are, so far



(13)



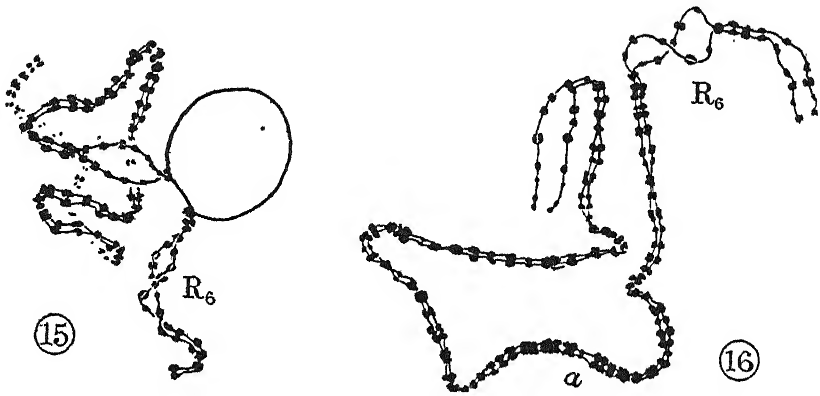
(14)

FIGS. 13 and 14—Pachytene in *F. imperialis* showing the unpaired parts of two pairs of chromosomes near their attachment to the nucleolus. Fig. 13, relational coiling beginning (same nucleus as photographs in fig. 30). Fig. 14, a later stage with coiling well developed (L_6 and R_6). $\times$ 2800

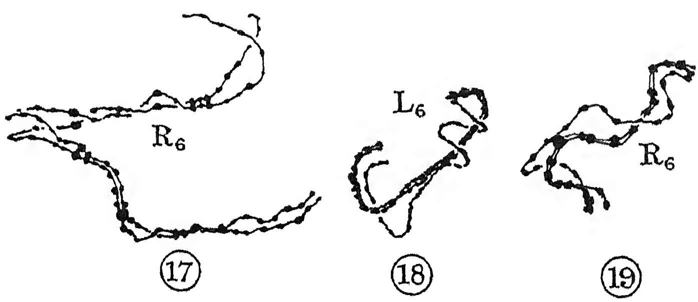
as their images with green light show, less than $0 \cdot 2$ μ in diameter, so that their division would scarcely be perceptible unless their products were separated by a similar distance. Division therefore has no doubt occurred in the smaller chromomeres without being distinguishable by visible light.

4—The Diplotene Separation

The separation of the paired chromosomes marks the beginning of diplotene, figs. 24 and 25. It is almost simultaneous for all the chromosomes in the nucleus. It starts in the proximal condensed region and passes gradually along the chromosomes, revealing the chiasmata and



Figs. 15 and 16—Late pachytene from the same nucleus of *F. imperialis*, showing non-pairing with division and relational coiling of unpaired intercalary segments, nucleolar and independent. × 2800

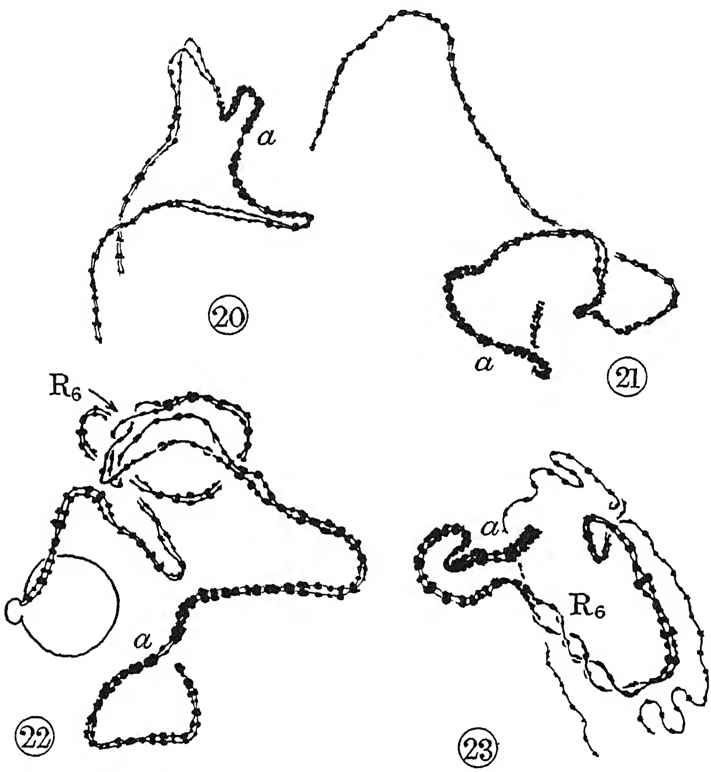


Figs. 17–19—The development of consistent relational coiling between distal paired and unpaired intercalary parts of chromosomes in the diploid *F. recurva* and the triploid *F. pudica*, without localization. × 2800

relational coiling by which they continue to be associated. The coiling, so far as it can be distinguished from the chiasmata, is seen to be consistent in particular arms of chromosomes, figs. 26, 27. Both are necessarily confined to segments between paired parts and it is easy in the species with localization to see the long distal regions of the chromosomes winding their separate ways through remote parts of the nucleus. These are the threads that have never been paired, fig. 28.

5—The Conditions of Incomplete Pairing

Comparison of the species with and without localized pairing is summarized in Table I and in fig. 29. It shows that there is no qualitative difference between their pairing behaviour. In all species pairing is incomplete. In those with localization it is arrested early. In those

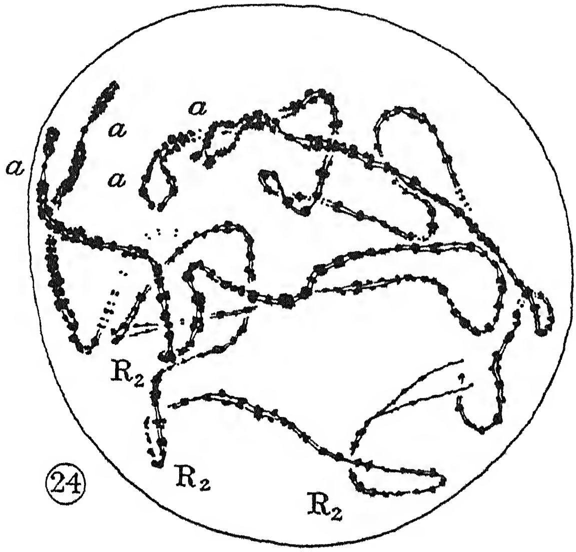


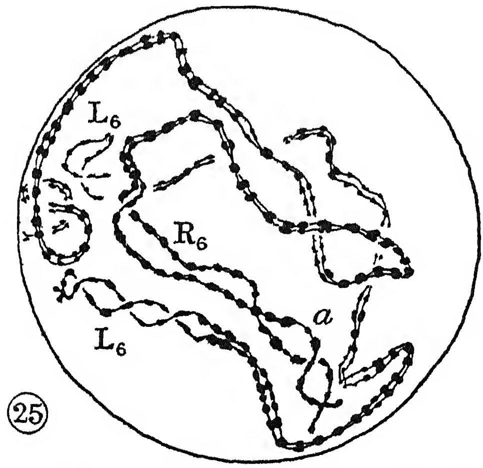
Figs. 20–23—Pachytene in species with localized pairing. Figs. 20–21, nearly complete pairing in the proximal parts of *F. pontica*; early pachytene with little coiling. Figs. 22–23, two bivalents from the same nucleus showing incomplete pairing with much relational coiling at late pachytene (R_6) in *F. oranensis*. × 2800

with almost complete pairing it is arrested only before the extreme ends and the parts near the nucleolus have paired, or it may even go on to completion.

The behaviour of the nucleolar chromosomes is peculiarly enlightening as showing the conditions of incomplete pairing. In every species of *Fritillaria* there are two or more pairs of chromosome bearing nucleoli—two pairs being usually developed at the secondary constrictions which

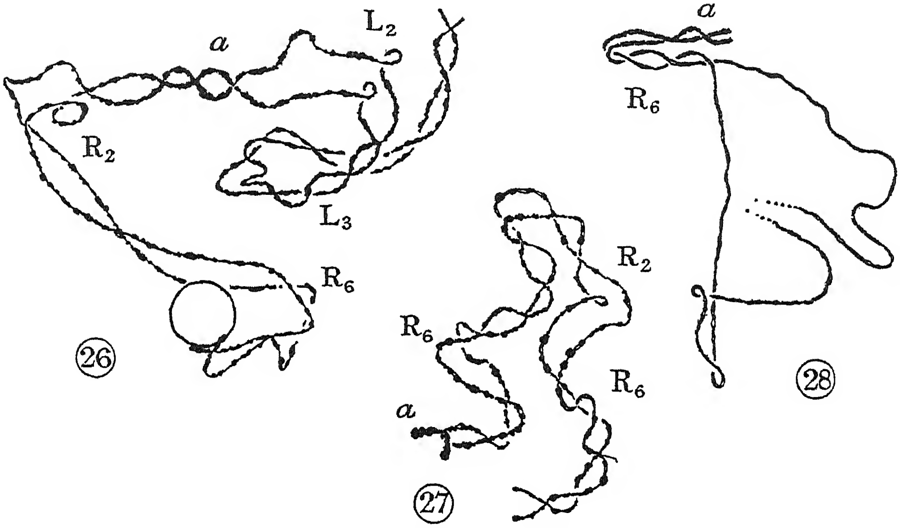
are so pronounced at mitosis (*e.g.*, in *F. ruthenica*, Darlington, 1929). A large part of the chromosome lies distal to these constrictions. The nucleoli no doubt arise in the way discovered by Heitz (1931). At the





FIGS. 24–25—The beginning of diplotene in *F. obliqua* with semi-localized pairing. Separation has begun at the spindle attachment in two bivalents while two others are still in a pachytene condition. One of these shows consistent relic coiling (R_2). Fig. 25—The same in *F. imperialis* with almost complete pairing. The two chromosomes show relational coiling as well as chiasmata. × 2800

leptotene stage they may be separate or fused. At pachytene the nucleoli of homologues are always fused, and in *F. imperialis* two pairs of nucleolar chromosomes are attached to a single fusion-nucleolus. It seems,



FIGS. 26–28—Diplotene separation complete; fig. 26 in *F. libanotica*, M chromosome; fig. 27, in *F. imperialis*; fig. 28, in *F. Meleagris* with localized pairing. Relational coiling not always distinguishable from chiasmata, but, so far as distinguished, consistent in particular arms and also consistent with relic coiling. × 2800

TABLE I—COMPARISON OF THREE TYPES OF CHROMOSOME PAIRING IN *Fritillaria*

	F. Meleagris L.	*F. Elwesii* Boiss	*F. imperialis* L.
Zygotene ..	Pairing begins near the spindle attachment.		
	Pairing ends while distal and middle parts are still unpaired or only intermittently paired.	Pairing ends while distal and middle parts are still only intermittently paired.	Pairing ends while some distal parts are still unpaired.
Pachytene..	Pairing often ends while parts of chromosomes near nucleoli are still unpaired.		
	Differential condensation begins near the spindle attachment so that the attachment chromomere becomes recognizable.		
	Pairing about 50% complete.	Pairing about 75% complete.	Pairing about 95% complete.
	Relational coiling develops between chromosomes in paired and intercalary unpaired parts.		
Diplotene..	Separation begins near the spindle attachment.		
	Little coiling, chiefly in proximal parts.	Much coiling, chiefly in middle parts.	Little coiling evenly distributed.
	Chiasmata localized in proximal parts.	Chiasmata chiefly in proximal and middle parts.	Chiasmata evenly distributed.

therefore, that fusion of nucleoli, those of homologues in particular, occurs frequently during the zygotene stage, no doubt as a result of the movement of the attached chromosomes and especially as a result of the pairing of the homologues. The parts of chromosomes near to their attachment to the nucleoli are then at a disadvantage. In the movement necessary for their pairing they are each separately shackled in the middle of an arm to a large body of high density (as we know from centrifuging experiments). We must expect that this handicap will delay their pairing where homologues have been far apart in the large *Fritillaria* nucleus. The observations show that they actually fail to pair in a proportion of nuclei. We are thus led to conclude that delay in pairing means inhibition of pairing.

This conclusion shows us why the distal parts fail to pair. Pairing begins in the proximal parts of the chromosomes. The distal parts are relatively delayed. They fail to pair in greater or less degree.

The parts that have failed to pair, whether distal or nucleolar, are seen at late pachytene to have divided. Is it this division which inhibits pairing ?

The unpaired threads at pachytene in trisomic maize are seen to have divided prematurely (Darlington, 1934). Here it cannot be supposed that the division determines the failure of pairing, since at any one point one of the three chromosomes, at random, fails to pair and prematurely divides. Pairing therefore leads to a postponement, or non-pairing to a hastening, of division. We may say that the threshold value of other conditions of division (such as the concentration of available materials in the medium) is higher for paired chromosomes in which the attraction for identical mates is satisfied or semi-satisfied than for unpaired chromosomes in which it is entirely unsatisfied.

This behaviour permits the assumption that the force which attracts the similar molecules or molecular groups of a sister homologue in pairing is also the force which determines the deposition of the molecules or molecular groups which constitute a daughter chromosome and thus produces " division " ; this assumption is the simplest one, since both association and reproduction are limited to pairs of identical particles. Association and duplication are then functions of the same attraction.

Whatever its theoretical significance in this direction, the division of unpaired chromosomes in a triploid makes it equally possible to suppose, *a priori*, that in any other case failure of pairing is antecedent to exceptional division of a chromosome, or consequent to it. We might therefore assume that in species of *Fritillaria* either of the two causal sequences, shown in Table II, leads to failure of pairing of the distal segments.

The first sequence is the one indicated in *Mecostethus*. It is at first sight the simplest and it has been adopted according to my suggestion by Huskins and Smith.* My present observations, however, make it impossible to accept this conclusion. So far from a precocity of the distal parts determining their behaviour, these parts are delayed in the whole series of events, including their division in the parts where they are paired; division occurs in the unpaired parts prematurely just as it does in trisomic maize. Assuming the second sequence, on the other hand, we can relate the differential behaviour of proximal and distal parts of the chromosomes to the precocity of the proximal parts in every respect in which they can be compared under equivalent conditions—their pairing at zygotene, their condensation at pachytene, their division and separation at diplotene. Furthermore, the assumption of this sequence enables us to understand the occurrence of variable and intermittent pairing as the result of variable spatial relationships in the nucleus demanding variable

TABLE II

A With differential time of division	B With differential time of pairing
1—Precocious division of distal parts.	1—Delayed pairing of distal parts.
2—Failure of pairing of distal parts.	2—Division of unpaired parts.
	3—Inhibition of pairing in these parts.

time for the association of the threads at zygotene. If two homologues happen to be lying close together at their distal ends, these ends will pair in time, even in *F. Meleagris*, and form exceptional chiasmata there, such as I have illustrated. (Newton and Darlington, 1930.)

And finally it enables us to see the different types of behaviour in *Fritillaria* as a related series. It is clear that the distinction between them arises only through the extreme length of the chromosomes—three to six times as long as in maize, for example. They begin to pair at their proximal ends. The pairing takes longer than in organisms with shorter chromosomes and smaller nuclei. In *F. imperialis* the precocity of the meiosis is sufficient to allow pairing to be completed except in the parts of chromosomes hampered by nucleoli. In *F. Meleagris*, on the other hand, the precocity is insufficient. The distal ends of the chromosomes divide before they can pair. They become effectively mitotic, fig. 29.

* The observations of these workers are fragmentary, but their illustrations can be reconciled with mine if it is assumed that they have taken pachytene to be leptotene and precocious condensation of proximal parts to be precocious splitting of what they believe to be distal parts.

From this study we learn that the precocity of the prophase which turns mitosis into meiosis is effective only if it gives enough time to the single chromosome threads to pair before they divide and can no longer pair. If the chromosomes are very long, the margin of time necessary is greater and the pairing will be restricted; if the pairing begins regularly in one region it will be localized in that region. Such a species is merely

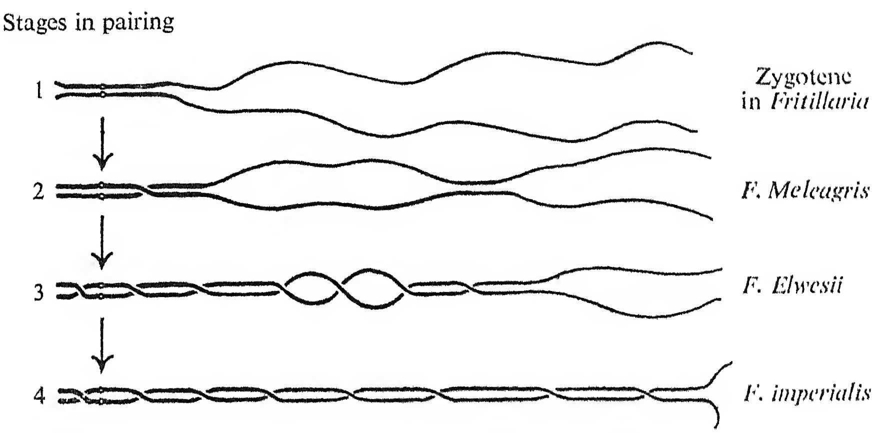


FIG. 29—Diagram to show the process of pairing in *Fritillaria* which is interrupted by the division of the unpaired parts at the second stage in *F. Meleagris* with extreme localization, at the third stage in *F. Elwesii* with intermediate localization, and at the fourth stage or when complete in *F. imperialis* with resultant free distribution of relational coiling 2, 3, and 4 thus represent the pachytene stage in the three species

semi-precocious. This simple deviation being responsible for localized pairing, its wide distribution in organisms with long chromosomes becomes understandable.

6—SUMMARY

In all species of *Fritillaria* the chromosomes pair first at zygotene, condense first at pachytene, and divide first at diplotene in the neighbourhood of the spindle attachment chromomere, which can be located at all these stages.

In all species pairing is incomplete and is particularly liable to fail in the parts attached to the nucleolus. These unpaired parts become relationally coiled and also divide during pachytene.

In some fifteen species, including *F. Meleagris*, pairing is interrupted while it is still far from complete. It therefore remains confined to the proximal regions, and localization of chiasmata results.

The interruption of pairing is presumably due to the observed division of the unpaired parts. Species with incomplete pairing are those whose chromosomes divide before they have time to pair; they are semi-precocious.

The bearing of these observations on the coiling seen at the later stages of meiosis and the mutual consistency of the evidence from successive stages will be considered in the next paper.

Description of Plate 3

Fig. 30—Photographs at two focusses of the nucleus illustrated in fig. 13. The attachment chromomere is faintly stained and its position is marked by a gap in the chromosome in the upper figure. × 2500

References

Darlington, C. D. (1929). 'J. Genet.,' vol. 21, p. 207.
—— (1930). 'Cytologia,' vol. 2, p. 37.
—— (1932, *a*). "Recent Advances in Cytology," London.
—— (1932, *b*). 'Biol. Bull.,' vol. 63, p. 357.
—— (1933). 'Cytologia,' vol. 4, p. 229.
—— (1934). 'Z. Indukt. Abstamm.-u VererbLehre,' vol. 67, p. 96.
—— (1935). 'J. Exp. Biol' (*in the Press*).
Heitz, E. (1931). 'Planta,' vol. 12, p. 774.
Huskins, C. L., and Smith, S. G. (1934). 'J. Genet.,' vol. 28, p. 397.
Newton, W. C. F., and Darlington, C. D. (1930). 'J. Genet.,' vol. 22, p. 1.

576 . 354 . 4

The Internal Mechanics of the Chromosomes
III—Relational Coiling and Crossing-over in *Fritillaria*

By C. D. DARLINGTON, John Innes Horticultural Institution

(*Communicated by Sir Daniel Hall, F.R.S.– Received February* 26, 1935)

1—INTRODUCTION

I have now outlined some of the considerations on which a study of this problem must rest. I have tried to show that the cyclical formation and resolution of spiral structure in the chromosomes at each mitosis entails a series of unforeseen changes which affect every aspect of chromosome behaviour. While the length of the chromosome regularly alternates between a maximum in the resting stage and a minimum at metaphase or anaphase, the degree of coiling of the chromosomes passes through a cycle of changes which lags behind this more elementary cycle. The complex conditions resulting from this lack of co-ordination can be explained by assuming that the changes in length are determined by the changes in internal structure which I have referred provisionally to the formation of a " molecular spiral." The uncoiling of the spirals formed in each mitosis is then delayed owing to a lag in the mutual adjustment of the chromosomes in the crowded telophase and prophase nucleus. The chromatids formed by the division of chromosomes in the resting stage are therefore coiled round one another in such a way as to compensate for the relic coiling of the chromosomes in which they jointly partake.

What kind of behaviour are we therefore to expect at meiosis? In this exceptional kind of division, prophase begins precociously, before the chromosomes divide, and this is a condition of their pairing (Darlington, 1932, and Part II). It also begins, according to the only available observations, before the maximum extension of the chromosomes is reached. In *Lilium* at the end of pachytene the chromosomes are about one and a-half times their length at an earlier stage of prophase (Belling, 1931). A similar increase is found in the marsupials, *Dasyurus* and *Sarcophilus* (Koller, 1935).

According to the present view, then, we must expect an uncoiling of the molecular spiral after the chromosomes have paired. This uncoiling

should lead to the coiling of the paired chromosomes round one another in the opposite direction to that of their internal spirals, unless their attractions allow them to slip.

In Part II, I have shown that such coiling develops at pachytene in the genus *Fritillaria*, a group of organisms which I have studied in every stage of meiosis and mitosis. The object of the present study is to find out more precisely the manner of this coiling. For this purpose it is of particular interest to study those species with localized pairing and chiasma formation, for here we may get an indication of the relationship of the coiling with the crossing-over which we now believe to determine the formation of chiasmata.

In these species I have already shown, in Part II, that the pairing of the chromosomes at the pachytene stage is complete only in the neighbourhood of the spindle attachment where it takes place first in all species. In the distal regions of the chromosomes it is intermittent or entirely lacking. The localization of chiasmata follows a localization of pairing at pachytene.

The chromosomes at the pachytene stage in *Fritillaria* are too long to allow of a quantitative description of their relationships and movements. To understand them we have to study the later, diplotene, stage and from this infer what we can of earlier behaviour. During diplotene the chromosomes become shorter and their general spatial relationships become clearer, thus providing a test of the interpretation of the earlier stage. The direction of coiling, if any is present, also becomes clear. For the study of this coiling at diplotene *Fritillaria* is particularly useful. The small amount of chiasma movement, terminalization, and the great length of the paired chromosomes, both make for stability of position. The spatial relationships at pachytene can therefore be inferred from the observations of those at diplotene with greater confidence than elsewhere.

To begin with such an inference, the degree of localization of chiasmata differs in different species. This can be taken as evidence that the degree of localization of pachytene pairing also differs. I have therefore studied the diplotene stage in species which differ in this way. *F. Elwesii* Boiss., the chief subject of study, has intermediate localization; the chiasmata are not strictly confined to the proximal regions. *F. Meleagris* L., with which it has been compared, has extreme localization and a lower chiasma-frequency (Newton and Darlington, 1930). A third species, *F. imperialis* L., has free distribution of chiasmata (Darlington, 1930, *b*). Pairing at pachytene, unlike that in species with localized chiasmata, is nearly complete (Part II).

2—Relational Spirals between Chromosomes at Meiosis

I—*Tabulation of Diplotene Stages in* F. Elwesii

a—Incomplete Diplotene (Part II)—Chromomere structure is still maintained and attachment chromomeres are still visible and marked by sharp constrictions. Precocious condensation in their neighbourhood is at a maximum. The paired chromosomes have separated in the proximal regions only (as in *Agapanthus*, Darlington, 1933). These region are lying chiefly towards one side of the nucleus. This is presumably due to a maintenance of the telophase orientation like that found by Belar (1929) in mitotic prophases and corresponding with the position of the centrosome in animals.

b—Early Diplotene, figs. 1–6—The separation of the paired chromosomes is complete, the chromomere structure is disappearing. The position of the attachment chromomeres is marked by a non-staining gap in the most condensed part of the chromosome. The chromosomes are always associated in this region by chiasmata or by relational coiling, which cannot be satisfactorily distinguished except in end-views. In their distal regions the partners may lie widely separated, figs. 1 and 5, and in these regions they are especially contorted.

Four pairs of chromosomes with sub-terminal attachment constrictions are seen to be attached to nucleoli at or close to their proximal ends. These nucleoli usually fuse to form one or two.

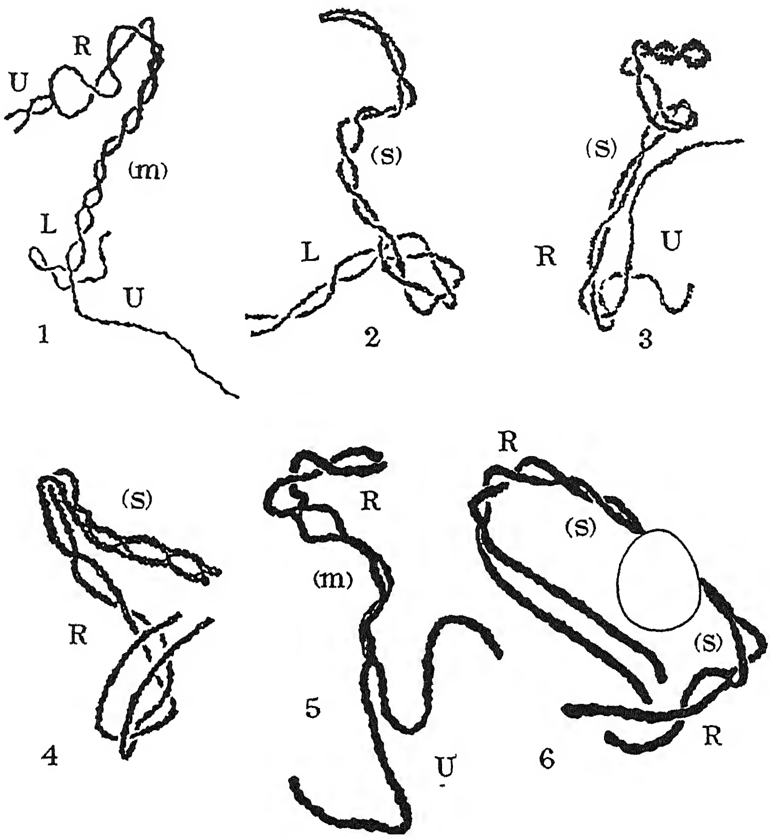
c—Mid-Diplotene, fig. 7—The chromosomes are thicker, shorter and more ragged in outline, perhaps owing to the assumption of the minor spiral.

Precocious proximal condensation, unlike that in *Agapanthus* and *Zea* (Darlington, 1934) has disappeared at this stage. Polarization of the nucleus is retained and the preservation of the general spatial relationships of the chromosomes indicates their stability.

The coils may be regularly distinguished from chiasmata and are seen to be less strictly localized. They occasionally lie between chiasmata but are more usually distal to them or are both intercalary and distal with respect to the chiasmata.

d—Late Diplotene, fig. 8—The chromosomes again have a more regular outline and are about one-third their pachytene length. (The minor spiral is presumably complete.) Their doubleness is less clear, except at chiasmata where chromatids exchange partners. The number

of chiasmata remains constant; the number of coils decreases by the rotation of the ends of the chromosomes. Both vary considerably from nucleus to nucleus.



FIGS. 1–6—Bivalents at successive stages of diplotene in *F. Elwesii.* Earliest stages, figs. 1–3, showing differential condensation persisting. Chiasmata not clearly distinguishable from overlaps in coiling. Later stages, figs. 4–6, with coiling and chiasmata distinguishable. The distal ends of chromosomes that are widely separated have not been paired, figs. 1, 3, and 5. R, right-handed relational spiral; L, left-handed relational spiral; *m*, medianly constricted chromosome; U, threads that have presumably been unpaired; *s*, submedianly constricted chromosomes. × 2000

e—End of Diplotene, fig. 9—The chromosomes have corrugations in their outline which sometimes correspond in number with the major spirals of metaphase. The increased rigidity of the more contracted chromosomes maintains successive loops between chiasmata at right angles and reduces coiling further, so that true coiling may be confused

with the chance relationship of unpaired arms or may be suppressed by the redistribution of strain between arms with opposite coils.

f—Diakinesis—The linear contraction of the chromosomes (formation of the major spiral) is complete and their outline is regular. Unpaired

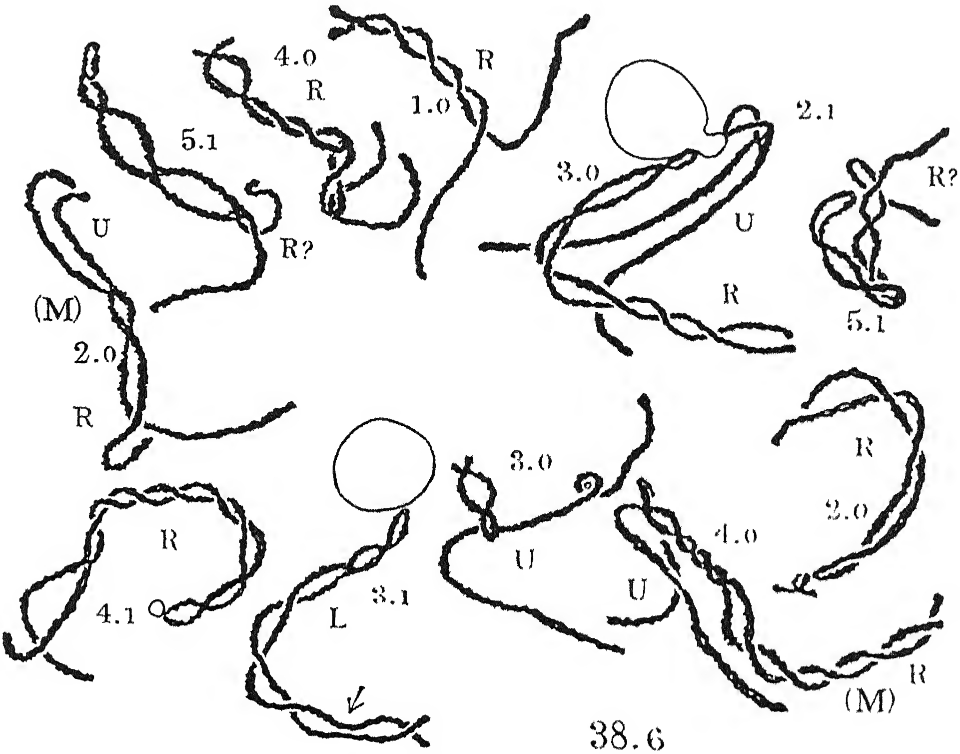


FIG. 7—Complete nucleus at mid-diplotene in *F. Elwesii*. The direction of the chromosomes, which has not been changed, shows polarization. Twelve pairs of chromosomes, two with median spindle-attachments (M), two chromosomes attached to one nucleolus. Of the 14 arms: 7 right, 2 doubtful right, 4 unpaired, 1 left. The arrow points to a region where a chiasma has perhaps been broken in smearing. One unpaired arm without relational coiling, U, is coiled by chance round a non-homologous chromosome shown in section. Numbers of total and terminal chiasmata given for each bivalent and below for the whole nucleus. × 2000

arms may still lie wide apart and the vestiges of relational coiling are still seen. The nucleoli disappear and leave secondary constrictions that are momentarily visible.

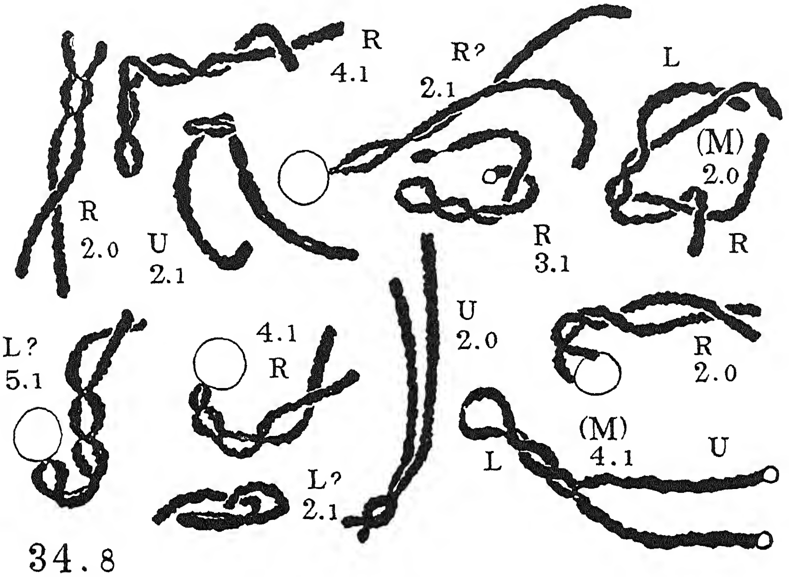


FIG. 8—Complete nucleus at late diplotene in *F. Elwesii.* Numbers of total and terminal chiasmata given for each bivalent, and below for the whole nucleus. Of the 14 pairs of arms: 6 right, 1 doubtful right, 3 uncoiled, 2 doubtful left, 2 left. × 2000

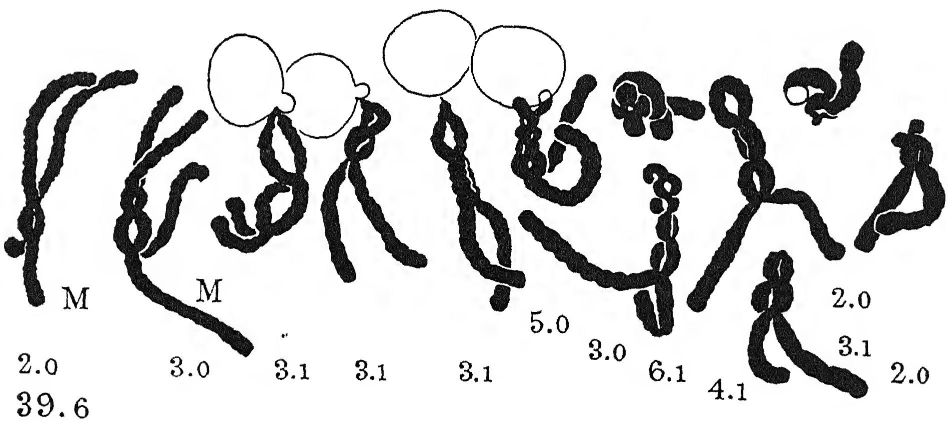


FIG. 9—Complete nucleus at early diakinesis in *F. Elwesii.* Total and terminal chiasmata given. Direction of coiling clear in only four pairs of arms, but cases of non-pairing still clear in three pairs. The same nucleolus is represented four times. × 2000

II—*The Relationship of Diplotene and Pachytene: Evidence of Localized Pairing*

In Part II, I have described and inferred three special properties of the paired chromosomes during the prophase pairing stages in the species of *Fritillaria* with localized chiasmata, all depending on a precocity of the spindle attachment region relative to the distal region, viz., (1) precocious pairing at zygotene, (2) precocious condensation at pachytene, (3) precocious division and resultant precocious separation at the beginning of diplotene. Associated with the first of these properties is the non-pairing or intermittent pairing—as the chances of spacing permit—of the distal regions of the chromosomes at pachytene. The second and perhaps the third are found equally in normal species of *Fritillaria* and in *Agapanthus* (Darlington, 1933).

The present study begins with the chromosomes completely separated, the second stage of diplotene. It is then found that the limbs of homologous chromosomes which are associated in the proximal region are widely separated in distal regions, fig. 1. Not only this, but between the separated homologues is lying the tangled mass of the other paired chromosomes, sometimes even coiled round them, fig. 7. These arrangements make it impossible that the separated limbs can have been paired during the pachytene stage. The regions separated at diplotene correspond with the unpaired parts of chromosomes described at pachytene in Part II.

A further distinction between the proximal and distal ends of the pairs gradually becomes evident as the prophase advances. The chromosomes are associated with one another, in the proximal regions where they are lying near together and pairing has been complete or nearly complete, in two ways; by coiling round one another, and by chiasmata.

The distinction between the overlapping of the coils and the crossing of the chromatids at chiasmata is usually uncertain at the earliest stages of diplotene. It is, however, clear even in these stages where the chromosomes are seen in end-view with changing focus. The chiasmata are then found to be even more strictly localized in the proximal regions than is the coiling. Coiling never occurs proximal to the first chiasma. It occurs perhaps equally often between two chiasmata or distal to the last chiasma. Comparison with the pachytene observations indicates that the regions where coiling occurs are those where pachytene pairing has been intermittent, for the coiling never extends to the ends of the chromosomes in the early part of diplotene.*

* Levan's description (1933) of the later prophase and metaphase stages in *Allium fistulosum* shows the behaviour of this species to be comparable with that of *Fritillaria Elwesii*. But numbers of chiasmata which he finds in the distal parts of the chromo-

III—*The Distribution and Redistribution of Relational Coiling*

When we compare the succession of stages during diplotene we cannot fail to be impressed by a subjective inconsistency between the figures of the chromosomes and the rates of movement that the comparison shows. The attitudes are the attitudes of a lively jig; the tempo is the tempo of a stately Nô dance, and it is the stateliness of the movement that makes its exact analysis possible. The spatial stability, like that shown by the persistence of relic spirals at mitosis, makes it possible to follow the succession of stages accurately.

The coiling of the chromosomes is variable in quantity at early diplotene in different chromosomes in the same nucleus and in the same chromosomes in different nuclei. But there is a definite relationship between the chiasmata and the coiling. In some pairs the whole region in which the chromosomes are lying near together has chiasmata at regular intervals. In others, however, there is a longer interval between certain chiasmata and between them the chromosomes are then coiled, the distance occupied by half a complete turn being about equal to an average distance between two proximal chiasmata not separated by coiling. The coils in any one arm of a chromosome are usually consistent in direction, either having a right-hand (corkscrew-wise) or a left-hand (anti-corkscrew-wise) twist. The coils are also of fairly constant amplitude.

There are exceptions to the constancy of direction, fig. 10, first bivalent. They consist always in a single distal coil in the opposite direction to the proximal coil and they occur in perhaps one in twenty of the pairs. The conditions from which they might arise are seen in the early diplotene, fig. 1, where the unpaired distal segments of the chromosomes lie in such a relationship as to produce the false effect of coiling after some contraction has taken place. The reason why this "false coiling" should usually be in the reverse direction from coiling will be considered later. There is no difficulty then in supposing that all true relational coiling derived from pachytene pairing is consistent in each chromosome limb.

The four complete nuclei that have been studied show a gradual diminution in coiling in successive stages, figs. 7–9 and 14. The twelve chromosomes, of which two have median attachments, have 14 limbs

somes at early diplotene have disappeared before metaphase without leaving any terminal chiasmata and without any change in the position of the proximal chiasmata. This interpretation is without parallel as it stands, but corresponds exactly with the behaviour in *Fritillaria* if under "chiasmata" we understand both true chiasmata and turns of the coiled partners, and it is in this sense that I would interpret Levan's observations.

whose coiling properties are potentially recognizable. In the earliest stage, mid-diplotene, eight of these are still well enough developed to be recorded with certainty; in the latest stage, early diakinesis, only four. The three earlier stages agree in showing reconcilable proportions of limbs with a right-handed twist, with a left-handed twist, and with no coiling. There is therefore reason to suppose that the coiling property of the arms of the individual chromosomes is constant as well as consistent.

It is necessary now to consider the direction of coiling of particular chromosome arms not only in order to test its consistency in different nuclei but also in order to follow accurately any changes that may occur between diplotene and diakinesis. Table I shows the directions of coiling in the two arms of one of the two M chromosomes (which cannot be distinguished in form of behaviour) in three nuclei at successive stages of diplotene, figs. 10 and 11.

TABLE I

	L/R	R/R	L/L
Stage 3	2	—	—
Stage 4	6	2	—
Stage 5	10	8	4
Total..................	18	10	4

These records are selected from a much larger number of observations in which coiling was not detectable in one arm-pair, the other being either left or right or also uncoiled. The conditions they represent might have been achieved if the original coiling, where it occurs in both arm-pairs, was left in one arm and right in the other, while where coiling occurred in only one arm-pair at the beginning, fig. 5, it developed later in the other arm-pair by a redistribution of strain from the coiled arm-pair and therefore consistently with it. That such a *redistribution* of coiling should occur is in fact inevitable. The observed diminution of the coiling during prophase can only take place by the relative rotation of the ends, accompanied by a redistribution of the coiling strain along the chromosome. Where one arm-pair is uncoiled—which is in about half of the cases at the earliest stage—it is bound gradually to develop, by redistribution, a coiling in the direction of the other arm-pair. It will be noticed that the exceptions so arising are predominantly right coils. This suggests that the longer arms of the M chromosomes are those with right-hand inter-chromosome coils.

The redistribution at diplotene suggests the possibility of an action of the paired on the unpaired parts of the chromosomes during the assump-

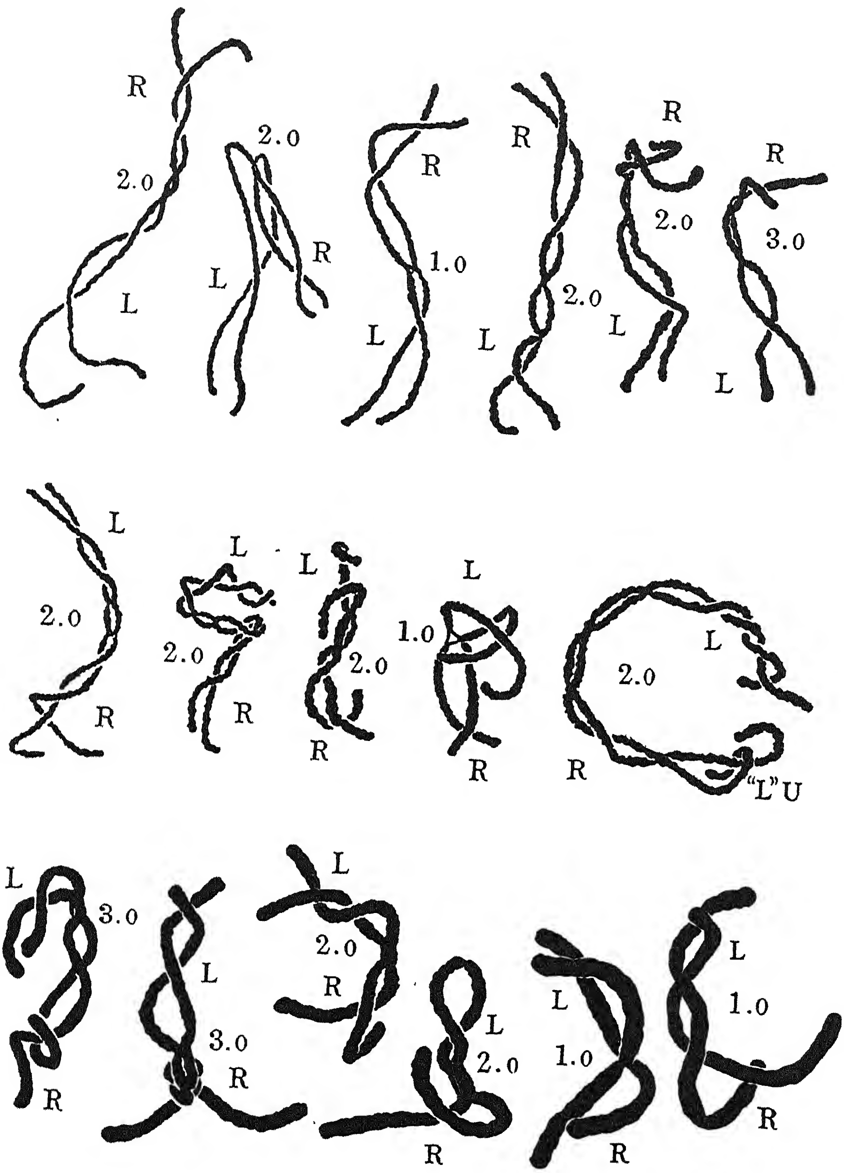


FIG. 10—Median bivalents from 17 different nuclei of *F. Elwesii*, from mid-diplotene to diakinesis. All have coiling consistently opposite in opposite arms. × 2000

tion of the coils at pachytene. The coils to be consistent must have been determined by a coiling of the chromosomes in their parts after association. It we coil two threads round one another after pairing them in the middle and leaving the ends free we have to drag these free ends round one another in the opposite direction to that of the intended coiling. This shows us how a false coiling of the free ends arises in the opposite direction to the true coiling and persists during diplotene. False

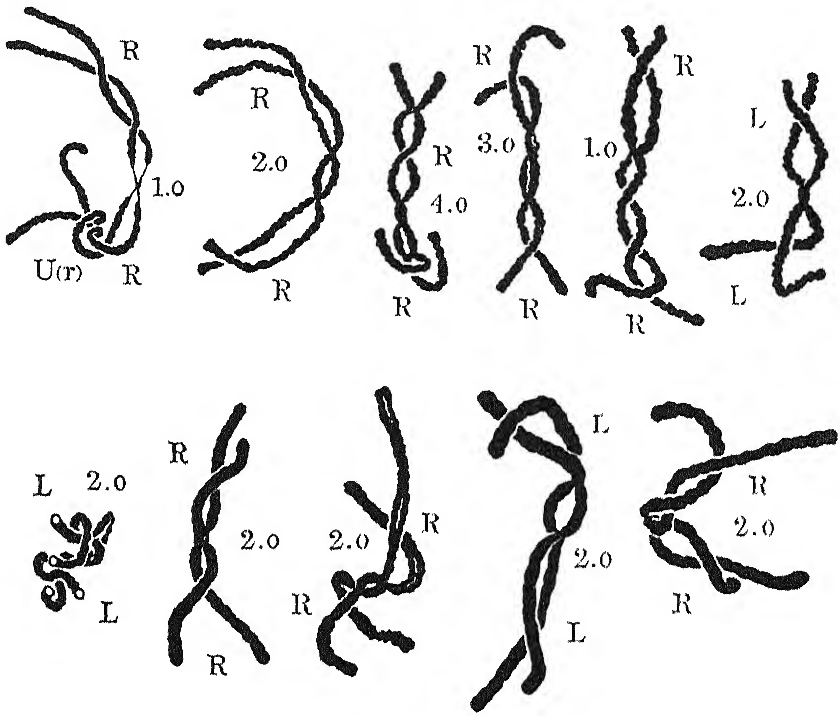


FIG. 11—Median bivalents from 11 different nuclei in *E. Elwesii* at late diplotene and diakinesis. These have coiling in the same direction, left or right, in opposite arms, as a result of redistribution of coiling-strain. *r*, relic coiling transferred from relational coiling (Type 7A). × 2000

coiling would, no doubt, be more frequent if it were not that the characteristics of such *free* relationships may be gradually lost during the long pachytene stage.

Another peculiarity of late diplotene is the appearance of individual coiling in both the free and relationally coiled arms of chromosomes. This new coiling is always consistent in a pair of arms, figs. 11 and 12. It is presumably due to another kind of redistribution, a transference of the strain on the relationally coiled parts to the unpaired parts; it is clearly not due to the survival of relic coiling after the long period of

adjustment during pachytene. To distinguish this form from original relic coiling it may be described as *transferred coiling* (Type 7A).

IV—*Relational Coiling in other Species*

F. Meleagris (Newton and Darlington, 1930) differs from *F. Elwesii* in having a lower frequency and a more strictly localized distribution of

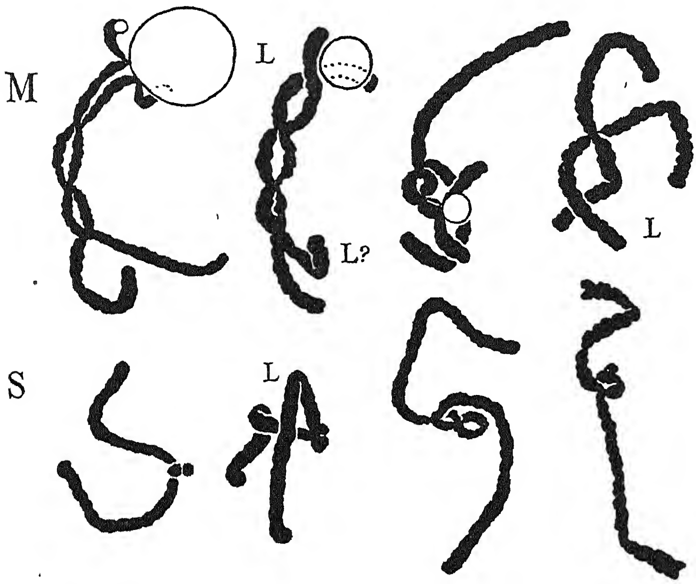


FIG. 12—Late diplotene bivalents from different nuclei of *F. Meleagris*. M, median bivalents showing rapid disappearance of attached nucleolus. S, subterminal bivalents showing transference of strain from relational coiling to individual coiling, giving " transferred spirals," also seen in the third and fourth M and in the first bivalent in fig. 11. × 2000

chiasmata. Thus in 11 pollen mother-cells of the two species I observed the following frequencies:—

F. Meleagris : 32, 29, 28, 26, 25 chiasmata or 2·3 per bivalent.
F. Elwesii : 39, 38, 36, 35, 34, 32 chiasmata or 3·0 per bivalent.

In *F. Meleagris* there is much less coiling at diplotene than in the other species, fig. 13. What there is in the M chromosomes also shows less consistency. In view of the explanation of inconsistency in these chromosomes given above, an increase of inconsistency would necessarily be found with a reduction in the totality of coiling.

F. imperialis (Darlington, 1930, *b*, and Part II), with almost complete pairing, has chiasmata and coiling evenly distributed along its chromo-

somes, but the coiling is so slight that it disappears before a late diplotene.

The comparison of the three species, *F. imperialis*, *F. Elwesii*, and *F. Meleagris* thus shows that the highest degree of coiling is found at diplotene in the species with an intermediate degree of localization.

3—Causal Analysis of Relational Coiling

I—*How Coiling can Occur at Diplotene : Intermittent Pairing*

Formerly it was often thought that the paired chromosomes were merely coiled round one another in the diplotene nucleus, whence this stage came to be designated " strepsitene." Recently the opposite point

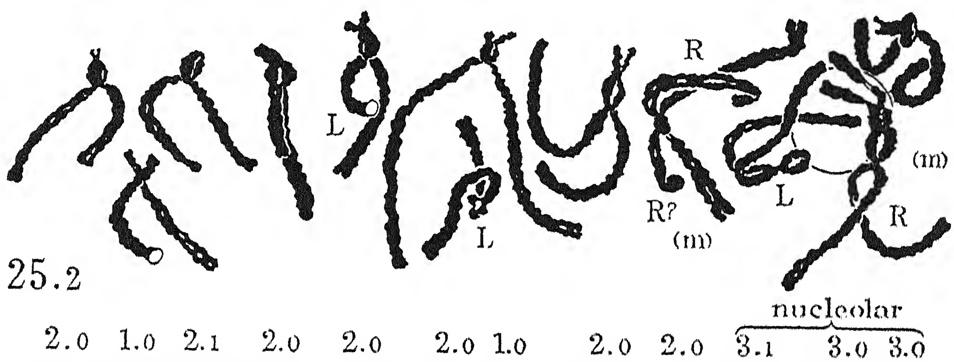


FIG. 13—Complete nucleus at late diplotene in *F. Meleagris*, showing less relational coiling than in *F. Elwesii*. × 2000

of view was expressed by Belling (1928), viz., that all overlaps of chromosomes were chiasmata at this stage and that strepsitene was in fact a misnomer. These extreme views are sound only in special cases. In certain aberrant plants with failure of pairing at metaphase no chiasmata are formed at diplotene, but the chromosomes remain in a temporary coiled relationship with one another (Richardson, 1935, on *Crepis*). In other plants, such as those species of *Lilium* and *Fritillaria*, with a high frequency of freely distributed chiasmata, there is little relational coiling at diplotene. On the other hand, it has recently been possible elsewhere to distinguish between coiling and chiasma-formation in the same pairs in *Anemone* (Moffett, 1932) and *Stenobothrus* (Darlington and Dark, 1932; illustrations). In species of *Fritillaria* with localized chiasmata it is particularly easy to make a sound distinction on account of the goodness of the fixation at the diplotene stage—a stage which formerly earned the name of " second contraction " on account of the usual collapse of the newly separated chromosomes under the action of fixatives.

We are now in a position to distinguish the conditions of origin of a coiling of the chromosomes at diplotene. At the earliest stages of diplotene, chiasmata and coiling are evenly distributed along those parts of the associated chromosomes which are lying near together and have been paired at pachytene. Chiasmata and coiling taken separately vary widely in frequency, but taken together they vary in accordance with the length of chromosomes lying close to one another. These circumstances suggest that the two are alternative relationships of associated chromosomes. The special distribution suggests that coiling is found instead of chiasmata in those regions which lie between paired segments although not paired themselves. Such *intercalary* unpaired segments result from the pairing at pachytene having been, as I have described it, intermittent, the paired segments distal to unpaired segments would then form chiasmata, or not, as in fig. 15, according to their length.

This conclusion is borne out by two kinds of observation. First, there is the finding of a lower amount of coiling with extreme localization, where evidently less intermittent pairing can be supposed to occur, and only the slightest coiling in *F. imperialis*, where pairing is complete or nearly so. Secondly, there are observations on triploids in which the conditions are analogous to those in *Fritillaria*. In such plants the pairing is intermittent, owing to the association at any one point being confined to pairs. As pachytene proceeds the unpaired third chromosome comes to lie closer to the other two. This has given some observers the erroneous impression that all three chromosomes are equally associated. Evidently, as it now seems, the third chromosome is merely drawn towards its partners by the development of relational coiling, and the essential association, which conditions chiasma-formation, remains in pairs.* If two of the three chromosomes pair along a considerable intercalary distance and form chiasmata in this part and also with the third chromosome at both ends, the third chromosome should retain its intercalary coiling which, since it will be prevented at diplotene from showing a reciprocal coiling with the other two by their greater strength, will be expressed as relational coiling of its chromatids, that is by an internal twist. This unusual figure I have illustrated in triploid *Tulipa* (Newton and Darlington, 1929, Plate III, fig. 8*d*) and it has been found in two more

* The torsion produced by molecular uncoiling is also evidently responsible for the "non-homologous pairing" described by McClintock (1933) from structural hybrids in *Zea*. Her photographs show relational coiling of the associated chromosomes. A twisted string will "pair" with itself in the same way if its ends are brought together —not satisfying an attraction but relieving a torsion.

cases by my colleague, Miss Upcott. These observations are particularly significant because they show that the development of pachytene coiling and its removal (where possible) by chiasma-formation is not peculiar to organisms with localized chiasma-formation but depends on the intermittent pairing, whatever its circumstances.

How are we to account then for chiasmata alone arising in the paired regions while coiling has developed in the intercalary unpaired regions? The simplest explanation is that the chromosomes have been coiled round one another at the end of pachytene throughout the associated regions—between the furthest paired points—for there is no force operating to twist the chromosomes round one another in the unpaired regions which is not also operating in the paired regions. The appearance of the threads suggests that this is so in *Fritillaria* and elsewhere. The coils must then have been replaced by chiasmata in the regions where the chromosomes have been directly paired, and have survived in the unpaired regions where chiasmata could obviously not have been formed to replace them.

This conclusion leaves us two problems to deal with: first the problem of how coiling can have arisen in regions between any paired parts of chromosomes at pachytene (whether the regions themselves are paired or not), and secondly, the problem of how the formation of chiasmata can remove this coiling in the paired parts.

II—*How Coiling can Arise at Pachytene: The Molecular Spiral*

Any theory of the internal structure and mechanics of the chromosomes must be co-ordinated with what we know of their external structure and behaviour. The external properties are necessarily better understood on account of the size of the elements involved. Any theory of internal mechanics which cannot be so co-ordinated is useless. The most critical tests are provided by our knowledge of the division of ring chromosomes, the coiling of chromosomes and chromatids in relation to one another, the terminalization of chiasmata, and crossing-over or chiasma-formation.

Consider first the division of the ring-chromosomes found in *Crepis* by Navashin (1930). The daughter chromatids separate freely as a rule. Occasionally they interlock, *i.e.*, one makes a complete revolution round the other. They never seem to divide so as to form one continuous ring, *i.e.*, one making a half-revolution round the other. In division therefore the two halves are distinct from one another. The sister chromatids do not break or cross over with one another. Nevertheless

we know that sister chromatids usually are equivalent in relation to genetic crossing-over, one breaking as frequently as the other. This suggests true division of one large thread into two equal small threads rather than the laying down of a daughter thread next to a larger parent one. Whatever the process may be, it occurs in such a way that the two threads if drawn out straight without torsion would lie parallel or with only one turn, since, at the time of reproduction during the resting stage the parent thread is lying in a spiral, the parent and daughter threads must have a compensating spiral relationship. This spiral we can, in fact, see at the beginning of prophase. The conditions can be imitated by winding part of two rings of thread round a rod bent into a circle and broken at one point. It is then seen that the two threads are wound round one another in the opposite direction to that in which they are wound round the rod. To give this relationship the cleavage of the chromosome must take place along a characteristic helical surface which may be described as the cleavage surface, *i.e.*, that surface which will divide the spiral chromosome in the resting stage, or whenever it is extended to the utmost, in such a way that the divided halves would lie parallel in one Euclidean plane if they were straightened without rotation of the ends.

Relational coiling of the chromatids should therefore compensate for their relic spirals and uncoil during prophase *pari passu* with the uncoiling of the relic spirals.

In many organisms the pairing of the threads in meiosis may occur simultaneously at different points. It must therefore follow much the same lines as the helical cleavage of the chromosomes in mitosis. The two associating threads will therefore have a spiral relationship with one another, compensating for any relic spiral that has survived the leptotene stage. This spiral will disappear as the relic spiral disappears and the paired chromatids will be lying parallel to one another.

But will this be the final condition in pachytene? Have the chromosomes paired along their cleavage surface so that when they divide their daughter chromatids will also lie parallel? This will depend on whether the molecular spiral is uncoiled to its fullest extent, on whether, in fact, the maximum length and minimum coiling which is reached in mitosis during the resting stage is reached in meiosis at the same time or later. The measurements referred to earlier show that the chromosomes continue to lengthen during pachytene, reaching their maximum at the end of this stage when they divide. Uncoiling of the molecular spiral therefore occurs beyond what is necessary for straightening the chromosome thread. According to the hypothesis of the molecular spiral, the chromosomes

should continue to uncoil during the pachytene stage and the pairing surface must lie in a helical relationship to the cleavage surface.

The change that will arise during pachytene in the relative internal and external situation of the paired chromosomes can then be represented by placing together two pairs of woollen threads (the relationship of each pair representing the internal situation within one chromosome), both twisted in the same direction, next to one another so that their touching fibres hold them in a stable lateral relationship throughout their length. If their ends are released both pairs of threads will uncoil and in so doing —according to the principle used in all spinning—will coil round one another in the opposite direction to the original coiling. But the new relational coiling that they establish between pairs of threads will not replace the old coiling within pairs. It will come into equilibrium with it.

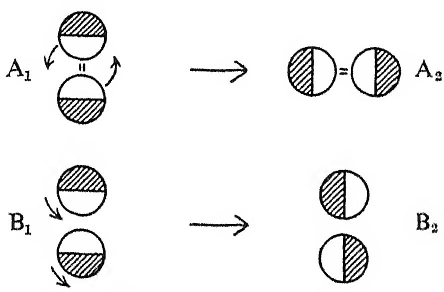


FIG. 14—Diagram to show how the individual rotation or twisting of two threads will lead to their relational coiling if they are paired by an attraction which is positionally specific, A, but not if they are paired by an attraction which permits a free change of lateral relationship, B

This model illustrates the mechanical principles involved when two or more threads which alone would lie straight come to take up a coiled relationship which is internally stable; first they must be held together by attractions which hold the parts in the same lateral relationship with one another and secondly they must be in a state of torsion, individually, in the same direction* and opposite to that in which they are relationally. The situation must depend on *lateral attraction* and *longitudinal cohesion*. These are two of the fundamental properties of chromosomes.

The lateral attraction must be defined more accurately. A stability in the transverse spatial relations of all the paired parts means a *positional specificity* in the attractions between these parts—chromomeres or genes, fig. 14. Such a specificity is to be expected from the properties

* Thus, if, as I suppose, this direction is individually and not relationally determined, the coiling of chromosomes in structural hybrids will be limited to similar pairs.

of molecular attractions demonstrated by experiment. Its existence as between parts of chromosomes is evidenced by the very fact of their relational coiling, for such coiling could only develop in opposition to the forces of longitudinal cohesion under the influence of a stable lateral attraction which was directionally specific.

The model also explains why we find chromosome coiling in *Fritillaria* and chromatid coiling in *Tulipa* under analogous mechanical conditions. The two kinds of coiling are reciprocal expressions of the same mechanical relationship.

And finally the model shows why it is that parts of chromosomes which are not paired themselves but merely lie between paired parts are coiled as effectively as if they were directly paired. Pairing at any two points not only attaches the chromosomes together at these points but also establishes one equilibrium system for all the intercalary region.

The model may therefore be taken to represent the essential mechanical conditions under which two threads will be coiled round one another. This means that the result of pachytene uncoiling of chromosomes will be to produce a system in which, when the chromosomes divide, they will be twisted round one another in the opposite direction to that in which they are internally twisted, fig. 15. There will be two equal and opposite types of relational coiling. Once established, the conditions that have determined this coiling will maintain it until either the lateral attraction or the longitudinal cohesion lapses. We have seen how the coiling can be removed gradually after the lateral attraction lapses at diplotene. We shall now find out how it can be removed suddenly when the longitudinal cohesion lapses at pachytene.

III—*How Chiasmata can Replace Coiling : Crossing-over*

We have to consider how chiasmata come to replace the coiling between the paired chromosomes. Chiasmata are exchanges of partner between paired chromatids. The pairs of chromatids on both sides of the chiasma are derived from single parental chromosomes. The separation on both sides is "reductional," and breakage and reunion of chromatids of partner chromosomes with an exchange of ends must therefore have occurred before the chromosomes could separate in this way. The reductional separation can be shown from the fact that relational coiling may be seen on both sides of a chiasma at diplotene, figs. 4, 7, 8, etc. The assumption that any of the pairs of chromatids was not derived from one parental chromosome would require that two such chromatids derived from the same chromosome were coiling round one another

each associated with a chromatid of a partner chromosome. This requires further untenable assumptions such as that the chromosomes divided and the chromatids recombined in their new association before

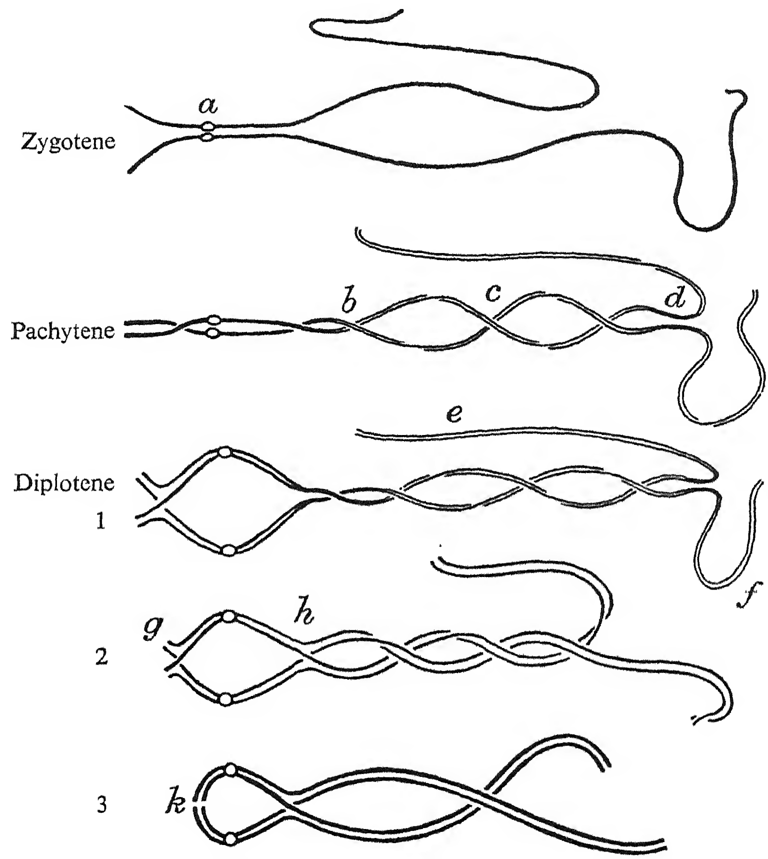


FIG. 15—Diagram to show the succession of events determining localized crossing-over and intermittent relational coiling in *Fritillaria*. The chromosomes divide in their unpaired parts at the end of zygotene, in their paired parts at the end of pachytene. The spindle attachment chromomere, *a*, does not divide till the next division. *b*, the end of the completely paired region. *c*, " intercalary " unpaired region which develops the coiling seen at diplotene. *d*, region that is paired distal to intercalary unpaired region. *e*, *f*, distal unpaired ends. *g*, *h*, two chiasmata formed in proximal paired region. *k*, the chiasma, *g*, terminalized. The relational coiling of chromosomes is right hand, that of chromatids left hand.

coiling began at the beginning of pachytene. The relational coiling therefore shows that crossing-over between chromatids of partner chromosomes is a condition of chiasma-formation. This I have shown before,

the demonstrations depending upon different kinds of assumptions according to the evidence used (Darlington, 1930 *a*, 1935). It is therefore crossing-over which replaces relational coiling.

The mechanical conditions determining crossing-over can now be defined with accuracy. Whatever views may be held as to the conditions permitting the development of the relational coiling seen at diplotene, one conclusion must inevitably be drawn from its presence as an alternative to chiasma-formation: the coiling represents a state of tension which is

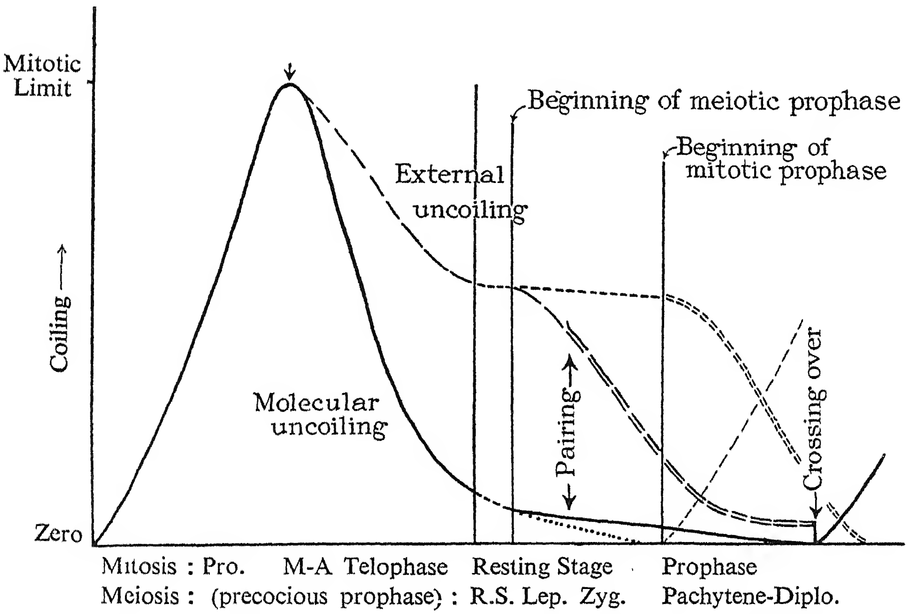


FIG. 16—Graph to show relationship of early prophases of meiosis and mitosis in regard to molecular coiling, and external coiling. Relational coiling, which arises to compensate for decrease of molecular coiling after pairing, is not shown. The arrest it determines in the decline of external coiling is broken by crossing-over (*cf.* diagrams in first article). For clearness the abscissa scale of meiosis is increased so as to give the minimum molecular coiling later than mitosis

relieved at once by chiasma-formation or gradually by uncoiling in the absence of chiasma-formation. These two methods of release follow the lapse of the two forces maintaining the coiling: longitudinal cohesion lapses when the chromosomes cross over; lateral attraction lapses when the chromosomes repel one another at diplotene.

A state of tension which is released when a given movement takes place must be held to determine that movement. Chiasma-formation depends on crossing-over, and crossing-over consists in the breakage of

two chromatids of partner chromosomes at opposite places and the reunion of the broken ends to form two new combinations corresponding with the old ones. Any change which reduces one kind of coiling will also, as we have seen, reduce the other, since they are reciprocal expressions of the same relationship. But the breakage and reunion required for crossing-over will allow the untwisting of both kinds of coil at once, for the broken chromatids change their relationships both with their sister chromatids and with one another. It will be capable of removing the coiling completely. The mechanical requirements of coiling therefore agree completely with the mechanical requirements of chiasma-formation on the assumption that the one is replaced by the other and that relational coiling determines crossing-over.

The consideration of the precise mechanism, the precise order of the events which constitute crossing-over, is necessarily more speculative. I have attempted to deal with it elsewhere in relation to its cytological and genetical consequences.

Summary

In *Fritillaria Elwesii* the chromosomes are paired at the pachytene stage of meiosis, completely in the proximal regions, intermittently further away from the spindle attachment, and not at all in the distal regions where they often lie wide apart. The spatial arrangement can be shown directly as well as by comparison with subsequent stages.

The chromosomes remain associated at the diplotene stage (when they repel one another) by chiasmata and by relational coiling. The chiasmata arise in the paired regions, the coiling arises in intercalary unpaired regions. In *F. Meleagris* with extreme localization there must be less intermittent pairing and there is less coiling.

Coiling is consistent at early diplotene within each arm-pair and for the same arm-pair in different nuclei.

The structures in the diplotene nucleus have a spatial stability which makes it possible to trace their movements accurately. Coiling gradually diminishes and changes by *redistribution* and *transfer* of strain. Traces of telophase polarization persist until diakinesis. The ends of chromosomes which have been unpaired at pachytene remain widely separated even at diakinesis. The positions of the chiasmata remain almost unchanged until metaphase.

The coiling represents a state of tension in the chromosomes which can only be due to opposition between the two forces maintaining the relative positions of the chromosomes: longitudinal cohesion and lateral attrac-

tion. The lateral attraction cannot be expected to cause relative rotation. It must be positionally specific and stable. But the longitudinal cohesion can do so since the chromosomes increase in length during the pachytene stage and therefore presumably uncoil their molecular spirals.

Such an untwisting, limited by positionally stable lateral attractions between the chromosomes, will set up at once an internal torsion in each chromosome and an opposite relational torsion between chromosomes in equilibrium with it. When the chromosomes divide, their chromatids will therefore be coiled round one another in the opposite direction to that in which the chromosomes themselves are relationally coiled. This will apply to unpaired intercalary regions as effectively as to paired ones.

The occurrence of coiling in unpaired regions and chiasmata in paired ones is therefore intelligible if we suppose coiling occurred throughout the chromosomes but was replaced by chiasmata in the regions which were paired. In most organisms this replacement will be nearly complete. Coiling will survive only in diploid organisms with partially localized chiasmata and in triploids.

Relational coiling often occurs on both sides of a chiasma. Such an arrangement demonstrates once more the principle that chiasmata result from crossing-over between two chromatids of partner chromosomes. It is also evident that no change other than crossing-over can remove both kinds of coiling at once. The tension set up by the opposition of longitudinal cohesion and lateral attraction is ended by the lapse in the lateral attraction at the end of pachytene when the chromosomes divide into chromatids. This results in a relief of the torsion either suddenly by the lapse of longitudinal cohesion in crossing-over and chiasma formation, or, in exceptional circumstances, by the gradual diplotene uncoiling.

A cytological consequence of these views is that failure of crossing-over and chiasma-formation (with failure of metaphase pairing) may be due either to failure of pachytene coiling (*e.g.*, *Zea*, Beadle, 1933) or to failure to release this coiling by crossing-over (*e.g.*, *Crepis*, Richardson, 1935).

The possible conditions of survival of coiling to the diplotene stage may then be classified as follows :—

(1) intercalary non-pairing
- (*a*) genotypically controlled, with localization;
- (*b*) structurally controlled, in triploids and inversion heterozygotes.

(2) failure of chiasmata to replace coiling in paired segments
- (*a*) entirely, in organisms with failure of metaphase pairing (*e.g.*, X strain of *Crepis*);

(*b*) partially, in normal organisms with a true "strepsitene" (*e.g.*, *Crepis* and *Stenobothrus*).

The genetical consequences have been considered elsewhere (Darlington, 1935).

References

Beadle, G. W. (1933). 'Cytologia,' vol. **4**, p. 269.
Belar, K. (1929). 'Z. Zellforsch. mikr. Anat.,' vol. **10**, p. 73.
Belling, J. (1928). 'Biol. Bull.,' vol. **54**, p. 465.
—— (1931). 'Univ. Calif. Publ. Bot.,' vol. **16**, p. 153.
Darlington, C. D. (1930, *a*). 'Proc. Roy. Soc.,' B, vol. **107**, p. 50.
—— (1930, *b*). 'Cytologia,' vol. **2**, p. 37.
—— (1931). 'Biol. Revs.,' vol. **6**, p. 221.
—— (1932). "Recent Advances in Cytology," London.
—— (1933). 'Cytologia,' vol. **4**, p. 229.
—— (1934). 'Z. indukt. Abstamm.-u VererbLehre,' vol. **67**, p. 96.
—— (1935). 'J. Genet.' (*in the press*).
Darlington, C. D., and Dark, S. O. S. (1932). 'Cytologia,' vol. **3**, p. 169.
Koller, P. Ch. (1935). 'Cytologia' (*in the press*).
Levan, A. (1933). 'Sv. Bot. Tidskr.,' vol. **27**, p. 211.
Moffett, A. A. (1932). "Chromosome Studies in Anemone. I—A new type of chiasma behaviour."
Navashin, M. (1930). 'Univ. Calif. Publ. Agr. Sci.,' vol. **6**, p. 95.
McClintock, B. (1933). 'Z. Zellforsch. mikr. Anat.,' vol. **19**.
Newton, W. C. F., and Darlington, C. D. (1929). 'J. Genet.,' vol. **21**, p. 1.
—— (1930). 'J. Genet.,' vol. **22**, p. 1.
Richardson, M. M. (1935). 'J. Genet.' (*in the press*).

611.018.2 : 612.799.7

Variation in Plumage Response of Brown Leghorn Capons to Oestrone

I—Intramuscular Injection

By A. W. Greenwood and J. S. S. Blyth (Institute of Animal Genetics, University of Edinburgh)

(*Communicated by A. S. Parkes, F.R.S.—Received March* 2, 1935)

Introduction

During a study on the ability of certain synthetic oestrus-producing compounds to induce a sex change in the feather pigmentation of the Brown Leghorn capon (Cook, Dodds, and Greenwood, 1934) it was noted that injections of the same preparation at varying times did not always cause a similar response in the feathers. That such irregular feather responses may be obtained limits the usefulness of the capon test, it was therefore necessary that more should be known of the reaction and an attempt made to determine some, at least, of the factors causing the variation in response.

An analysis of our previously collected data on the action of oestrone confirmed the opinion of Lillie and Juhn (1932) that the reactivity of the feathers of test animals varied considerably, but there was no evidence to show whether variable responses to the same stimulus occurred in one and the same individual or, if this were so, whether the changes behaved in a definite manner correlated with a varying environmental factor, such as season for example.

Three possible factors are suggested that might be presumed to modify the extent of the reaction in the individual: (1) the age of the bird; (2) the season of the year at which the injections are made, and (3) the body weight. The main investigation therefore took the form of a large scale experiment, repeated four times within a year, in which birds of varying age were used. A sufficiently wide range of difference in body weight occurred in the stock to allow of the determination of any relation it may have to the varying responses encountered.

Material and Technique

Altogether 87 Brown Leghorn castrated males were used. These were of varying age from 7·5 months to 5·5 years at the beginning of the

study; the youngest birds were a group of 55, 7·5 to 8·5 months old, while the remainder (32), considered as the mature group, were 1·5 to 5·5 years old.

The material for injection has been drawn from a single preparation of crystalline oestrone. It was provided through the generosity of M. André Girard, of Paris, to whom our thanks are due.

For the first series of injections, which were carried out on the 55 young capons only and were somewhat of a preliminary nature, the solution was prepared direct from the oestrone crystals in 50% alcohol; oestrone was present to the amount of 0·15 mg per cc but, as it barely went into complete solution, the actual concentration was slightly below this.

To obviate the difficulties encountered in this first experiment, which would inevitably introduce a variable into the tests, subsequent solutions were prepared differently; sufficient oestrone for all the experiments was dissolved in absolute alcohol to a concentration of 0·83 mg per cc and kept in a sealed bottle to avoid the possibility of evaporation. From this, stock solutions have been made up, as required, for each set of injections and in all injections the concentration used has been 0·25 mg/cc in 60% alcohol. In this medium the oestrone remains completely in solution, and the higher alcohol content appears to have no deleterious effect on the test animals.

Growing feathers are necessary for the test, and while these are usually to be found on capons at most seasons of the year, the variability of their distribution makes them unsuitable for this purpose. In each bird, therefore, a more or less homologous series of feathers was plucked about 2 weeks before the time of injection, thus provding a comparable group of uniform age. The feathers were removed from a transverse row on one side of the breast immediately behind the furcula; no attempt was made to take exactly the same row in each bird for the slight variation in feather size in adjacent rows is well within the limits obtained in individuals of different body size.

The exact time relations of plucking and injections are given in Table I. Injections were commenced when the feathers had grown sufficiently to pierce the skin but had not yet emerged from the sheath. In both the original and subsequent concentrations the daily dose given was 1 cc; it was administered on two consecutive days and then this procedure was repeated following an interval from 3 to 6 days. It was found that the 24-hour period between consecutive injections did not give rise to a discontinuous response although a gap of three or more days resulted in the return of the melanin in the feather, characteristic of the male, in varying amounts. Figs. 2–9 demonstrate the range of variation in this

TABLE I

Group	No. of birds	Date plucked	Time interval days	Injections 1st	Injections 2nd	Time interval days	Injections 3rd	Injections 4th	No. of test
Young capons	55	10.12.33	11	21.12	22.12	3	25.12	26.12	Prelim.
	48	24.1.34 25.1.34	13 12	6.2	7.2	4	11.2	12.2	1st
	51	25.4.34 26.4.34	15 14	10.5	11.5	6	17.5	18.5	2nd
	51	2.11.34	12	14.11	15.11	5	20.11	21.11	3rd
Mature capons	32	29.1.34	13	11.2	12.2	4	16.2	17.2	1st
	24	12.5.34 13.5.34	16 15	28.5	29.5	6	4.6	5.6	2nd
	26	5.11.34	12	17.11	18.11	5	23.11	24.11	3rd

respect and in fig. 13, a transverse row of feathers showing extreme restriction of the melanin is seen. Injections were made deep into the pectoral muscles of the breast, the first being introduced on the left side, the second on the right on each occasion. The feathers were then allowed to grow almost to maturity before being plucked.

Results

The feather reactions obtained in the first preliminary test with young castrates were very variable, and clearly illustrated a differential response on the part of individual birds to an identical stimulus. The reactions varied from a good response, where two complete bars of red pigment were formed across the vane of the feather, to the absence of the pigment. Out of 55 young capons treated there were 11 birds in which no reaction was obtained. It is evident that the amount of oestrone injected was in the region of the minimal dosage required to produce a characteristic response in an unselected population of birds, and the results are to be considered unsatisfactory because of the relatively high proportion of birds not responding to the treatment, indicating that the complete range of individual variation is inhibited in expression. It was decided therefore to increase the amount of oestrone injected in order that the whole range might be exposed. The increase, however, was not of such a magnitude that the maximum responses could have been produced by any lower dosage because individual mature birds gave a much better response to the same dose. With a concentration of 0·25 mg per cc no bird in any of the succeeding tests gave a completely negative reaction.

In order to make a comparison of the extent of the reaction in different birds it was necessary to attempt a classification of the magnitude of the responses obtained; these varied from an axial spot of red pigment on the rachis to a wide band stretching from one side of the feather to the other. Between these, all intermediate grades are shown. The technical difficulties in the way of making an absolute determination of the amount of red pigment present led to an attempt at arbitrary classification. The reactions were divided into five classes as follows: negative, rachis spot, narrow band, medium and wide band of pigment, Table II. Figs. 1–4

Table II—Score Value of Reaction

No reaction	0
Rachis spot	1
Narrow bar	2
Medium bar	3
Wide bar	4

FIG. 1—Rachis spot; FIG. 2—Narrow bar; FIG. 3—Medium bar; FIGS. 4 & 5—Limits of variation in young castrates of wide bar

Score value 1; 2; 3; 4

(First colour band only)

Range of variability of response. Young castrates

illustrate, with reference to the first reaction in the feather only, typical responses on which the classification was based. Figs. 4 and 5 represent the limits of variation in the *wide* band of pigment found in the young

Fig. 6 1932 castrates Fig. 7 Fig. 8 1929 castrates Fig. 9

Range of variability of response. Mature castrates

castrates, while the complete range in response in the youngest and oldest birds in the group of mature castrates is shown in figs. 6 and 7, and figs. 8 and 9, respectively. To the five classes of reaction a separate numerical

value has been given in order to facilitate the interpretation of the results. As with all arbitrary classification it is only relatively exact since the classes are not sharply differentiated, but considerable care has been exercised to obtain a reasonably correct interpretation of the amount of the reaction.

Effect of Age on Response

The data for comparison are those obtained from young and mature castrates injected in February, May, and November. Although 55 birds of the young group were used in the preliminary test, and 32 mature birds in the February test, owing to the employment of a number of birds in other experiments only 44 of the first group and 20 of the second were available for all the subsequent tests. Only data from birds that have undergone the whole series of experiments will be considered and compared. From Table III it will be seen that in the young birds when the score value was calculated, individual reactions at the first test ranged from 2 to 8 for the two sets of injections. (The range of values possible vary from 0 where both injections in the test gave a negative response to a maximum of 8 when two wide pigment bands were obtained on the feathers.)

Table III

Young castrates				Mature castrates			
No. of birds	Score test 1 (Feb.)	Average score test 2 (May)	Average score test 3 (Nov.)	No. of birds	Score test 1 (Feb.)	Average score test 2 (May)	Average score test 3 (Nov.)
3	2	5·0	4·3				
11	3	5·2	5·2				
11	4	6·4	6·3				
11	5	6·6	6·7	2	5	7·5	8·0
5	6	7·0	7·4	4	6	6·0	6·5
3	7	8·0	7·7	4	7	7·7	7·2
0	8	—	—	10	8	7·8	7·9

Of the total of 44 young castrates, 25 birds, 57% of the population, had a score value of less than 5 at the first test, while none of the mature birds gave such a small response; in addition although none of the young birds gave a maximum reaction at first test 50% of the mature group did so. It appears then that the age of the birds at injection is a major factor in determining the magnitude of the responses and this is borne out by the findings in the second and third tests; the average values of the reactions in these were obtained for the classified groups at first test, and a general all round increase in the magnitude of the reaction

is shown in the group of young castrates where it will be seen that those giving a poor response at the first experiment (score value from 2 to 4) exhibit the greatest increase in response at the second and third test. In spite of this, however, it should be noted that *the increase has not resulted in the elimination of the reaction classes*; those birds giving the poorest responses at the first test for instance, still give the poorest responses at subsequent tests, and in fact it was found that although the general level of response had increased, its character in individual birds remained a constant feature of those animals.

The mature castrates in their second and third tests gave no such clearly marked indications of an increased reactivity to the stimulus, and apart from the two birds giving the poorest response at the first test, were fairly uniform throughout.

Since the young castrates at the time of the third test had just attained an age similar to that of the youngest of the mature group at their first test, it might have been expected that the scores of the former would have

TABLE IV

	Test 1							Test 2							
Score:	2	3	4	5	6	7	8	2	3	4	5	6	7	8	No. of birds
Group															
Young ..	3	11	11	11	5	3	0	0	0	4	11	9	10	10	44
Mature ..	0	0	0	2	4	4	10	0	0	1	0	2	4	13	20

	Test 3							
Score:	2	3	4	5	6	7	8	No. of birds
Group								
Young ..	0	2	3	10	9	8	12	44
Mature ..	0	0	0	0	3	5	12	20

approximated more closely to those of the latter. That this is not so evidently depends on the fact that the larger population in the young group has exposed a wider range and different distribution of individual behaviour. This is made clear when the birds are grouped according to their score value in all three tests, Table IV. Discarding the first test for the young birds where the effect of immaturity is marked, it is found that for the next two, the number of individuals giving the greatest response is roughly the same as that obtaining throughout all the tests with the mature group; this distribution naturally is reflected in the

average score of young and mature birds at each test, for while half of the population of mature birds exhibit a maximum reaction, only about a quarter of the individuals comprising the population of young castrates do so. That the grouping of the birds is fairly constant in the last two tests of the latter argues that they have reached an age where maximum responses are obtained.

Effect of Season on Response

When considering the possible effect of season on the reaction this is complicated in the group of young castrates, by the factor of age. If the results of the first test on this group are ignored on this account, and only those obtained in May and November compared, it will be seen that there is no difference in the average response although the experiments were performed at widely differing seasons, Table V. The behaviour of the mature birds at the second and third tests also shows no deviation in the average amount of response although the result of the first test (February) is somewhat lower than the succeeding ones. This difference is almost entirely accounted for by the marked increase in response by the two birds giving the lowest grade of reaction at the first test; if these are omitted (bracketed figures) the response of the remaining birds shows little deviation in any of the three tests.

Table V

Age group	No. of birds	Average reaction value		
		Feb. test	May test	Nov. test
Young castrates	44	4·3	6·2	6·2
Mature castrates	20	7·1	7·4	7·4
	(18)	(7·33)	(7·39)	(7·39)

In general then it may be said, and this is supported from observations on isolated experiments at different times, not reported here, that season plays no significant part in determining the magnitude of response to an identical stimulus.

Effect of Body Weight on Response

Since the amount of oestrone injected into each bird was the same any relation existing between body weight and the magnitude of the response should be apparent if, in the population at the time of the experiment, the differences in body weight are sufficiently large. From Table VI

it will be seen that body weight in the experimental birds varied from a minimum of 1800 gm to a maximum of 3750 gm. If body weight were a factor in determining the efficiency of the dosage employed it is to be expected that the heaviest birds (receiving therefore the smallest amount of oestrone per unit of body weight) would exhibit a lower degree of reaction than the lightest birds. That this is not so can readily be seen. Although it has been shown that the average response by the mature birds is considerably greater than that given by the young castrates, the average body weight of the former group exceeds that of the latter by more than 400 gm. Moreover, the heaviest bird in the population,

TABLE VI

Grouped score values for three tests	No. of birds	Average body weight in gm	Class range of body weight in gm
Young castrates—			
10–13	10	2400	1800–2950
14–16	12	2365	1850–3150
17–19	11	2460	1800–3000
20–23	11	2560	2000 3000
Total..........	44	2445	1800–3150
Mature castrates—			
16–19	4	2940	2600–3150
20–23	8	2920	2700–3100
24	8	2895	2650–3750
Total..........	20	2910	2600–3750

3750 gm gave a maximum response while the lightest ones gave only indifferent responses. The birds have been classified according to the total number of points scored in the three tests and it is seen that the members of any one group exhibit a wide variation in body size, also there is no suggestion from these figures of a correlation between increased amount of response and decreased body size. The results clearly illustrate that no direct relation exists between the body weight of a bird (within the limits shown by the population in these experiments) and the amount of the reaction obtained, or rather that any relation to body weight is completely overridden by the effects of other variables conditioning the degree of response.

Individual Variation in Response

It has been shown that not only is age a factor in the variability in response obtained when a population consisting of birds of different ages is subjected to an identical stimulus but also that characteristic individual responses may be exhibited, different birds showing fairly constant different susceptibilities to the standard dose. In the former case the variation may be considered at present as resulting from the action of physiological factors while the latter indicates the presence of genetic differences controlling the degree of response. Still another type of variability is demonstrated when the responses obtained in the individual feathers of single birds are compared. Within the limits of the material two classes may be studied: (*a*) variability in an apico-basal direction along the individual feather, and (*b*) variability medio-laterally along a row of feathers in the breast tract.*

(*a*) *Apico-basal Variation*—It was seen that, when two injections of similar amounts of hormone were made with an intervening period of from 3 to 6 days between them, generally the response to the first injection was much more clearly marked in the feather than the second, figs. 3 and 4. A comparison of the magnitude of reaction to the first and second injection series is given in Table VII where it is demonstrated that measurable differences in response occur in both the young and the mature groups and that the trend is characteristically the same in all tests thus suggesting that there is a decreasing reactivity to oestrone in an apico-basal direction in the feather.

But although this is the conclusion that may be drawn from the average scores, a critical examination of the records shows that it cannot be held to be a universal law. A detailed analysis of the types of reaction occurring in individual birds is given in Table VIII*a* and *b*. In the former the reactions are classified for each test separately and it will be seen that not only does a large proportion of the birds give reactions in which the second pigment band is less in extent than the first on the same feather in all experiments, but the number of birds exhibiting this type of response also remains relatively constant. However, when the responses of the individual birds in all the tests are followed it is seen, Table VIII*b*, that only 24 birds out of a total of 44, and 13 mature birds out of 19 gave *consistently*, at all tests a much better reaction to the first series of injec-

* For a comparison with the findings of Lillie and Juhn (1932), it should be made clear that the relations about to be described exist whether considered as the width of the pigment bars along the rachis or along the barbs.

TABLE VII

Young castrates	No. of birds	Rachis spot	Narrow bar	Medium bar	Wide bar	Average score	% increase in score
Injection 1—							
February test	44	4	21	10	9	2·5	—
May test	44	0	4	16	24	3·5	40·0
November test	44	0	5	13	26	3·5	Nil
Total........	132	4	30	39	59	3·2	
Injection 2—							
February test	44	16	24	2	2	1·8	—
May test	44	1	20	10	13	2·8	55·5
November test	44	2	20	10	12	2·7	−3·6
Total........	132	19	64	22	27	2·4	
Mature castrates							
Injection 1—							
February test	20	0	0	3	17	3·8	—
May test	20	0	0	1	19	3·9	2·6
November test	20	0	0	0	20	4·0	2·6
Total........	60	0	0	4	56	3·9	
Injection 2—							
February test	20	0	5	5	10	3·2	—
May test	20	0	3	4	13	3·5	9·4
November test	20	0	3	5	12	3·4	−2·8
Total........	60	0	11	14	35	3·4	

tions than to the second; of the remaining birds the majority gave variable responses at the different tests. This deviation, from what might be considered, from the frequency of its occurrence, as the normal behaviour of the feathers is sufficiently large to prevent, in the absence of explanation, the formulation of an hypothesis to account for the decreased reaction proximally, observed in the majority of the feathers.

It might be suspected that the atypical reactions could most readily be explained by reference to differences in the amount of substance injected, resulting from faulty technique, and if this were so then their occurrence need not affect the validity of a general hypothesis relative to the decrease in reactivity of the feather, from apex to base, to the hormone. It is doubtful, however, that such an explanation can be

considered in view of the fact that the utmost care was practised to ensure that the correct amount of fluid was injected each time.

(*b*) *Medio-lateral Variation*—In addition to the difference in amount of reaction noted at different levels in the individual feathers, the data from the first two tests suggested that changes in the amount of response to the stimulus varied also according to the position of the feather in relation to the median line of the bird. In the third test therefore the

TABLE VIII*a*

	Young castrates				Mature castrates			
	Test			Total	Test			Total
	1	2	3		1	2	3	
2nd bar < 1st	33	32	36	101	16	15	15	46
2nd bar = 1st	8	4	4	16	0	2	1	3
2nd bar > 1st	3	8	4	15	3	2	4	9
Total observations	44	44	44	132	19*	19*	20	58

TABLE VIII*b*

	Young castrates	Mature castrates
2nd bar < 1st	24	13
2nd > 1st	1	2
2nd = to 1st	1	0
2nd = and < 1st	7	3
2nd = and > 1st	1	0
2nd < and > 1st	7	1
2nd = < and > 1st	3	0
Total No. of birds	44	19*

* One of the mature birds could not be included in this classification because in two of the tests a continuous effect was produced in the feathers in spite of the time interval between the two sets of injections.

preliminary plucking of feathers was such that ten feathers in a single row, extending from the keel laterally, at approximately the same level in all birds, were removed, in order to determine whether definite changes in response along this line were a feature of the reaction. The findings for both groups of castrates are shown in Tables IX and X.

From Table IX it is seen that more than 50% of the young birds (25) give reactions in which the first band of red pigment tends to increase in amount passing from median ventral to lateral edge of a single row

of breast feathers, and in these the second band either shows no marked change in the amount of pigment present, fig. 10 or else it decreases progressively, fig. 11. In only two birds was an increase noted in the second band when the first showed definite increases. Four birds showed no change in the amount of pigment in either band and the remaining group of ten birds, although showing no change in the size of the first band, gave a definite decrease in the second.

TABLE IX

Median to lateral changes in response	No. of birds: Young castrates	No. of birds: Mature castrates
Increase injection 1; increase injection 2	2	11
Increase injection 1; no change injection 2	12	6
Increase injection 1; decrease injection 2	13	1
No change at either injection 1 or injection 2 ..	4	0
No change injection 1; decrease injection 2	10	1
Total No. of birds	41*	19*

* Three young and one mature bird with irregular distribution of pigment at the edges of the bars, rendering classification difficult, were not included.

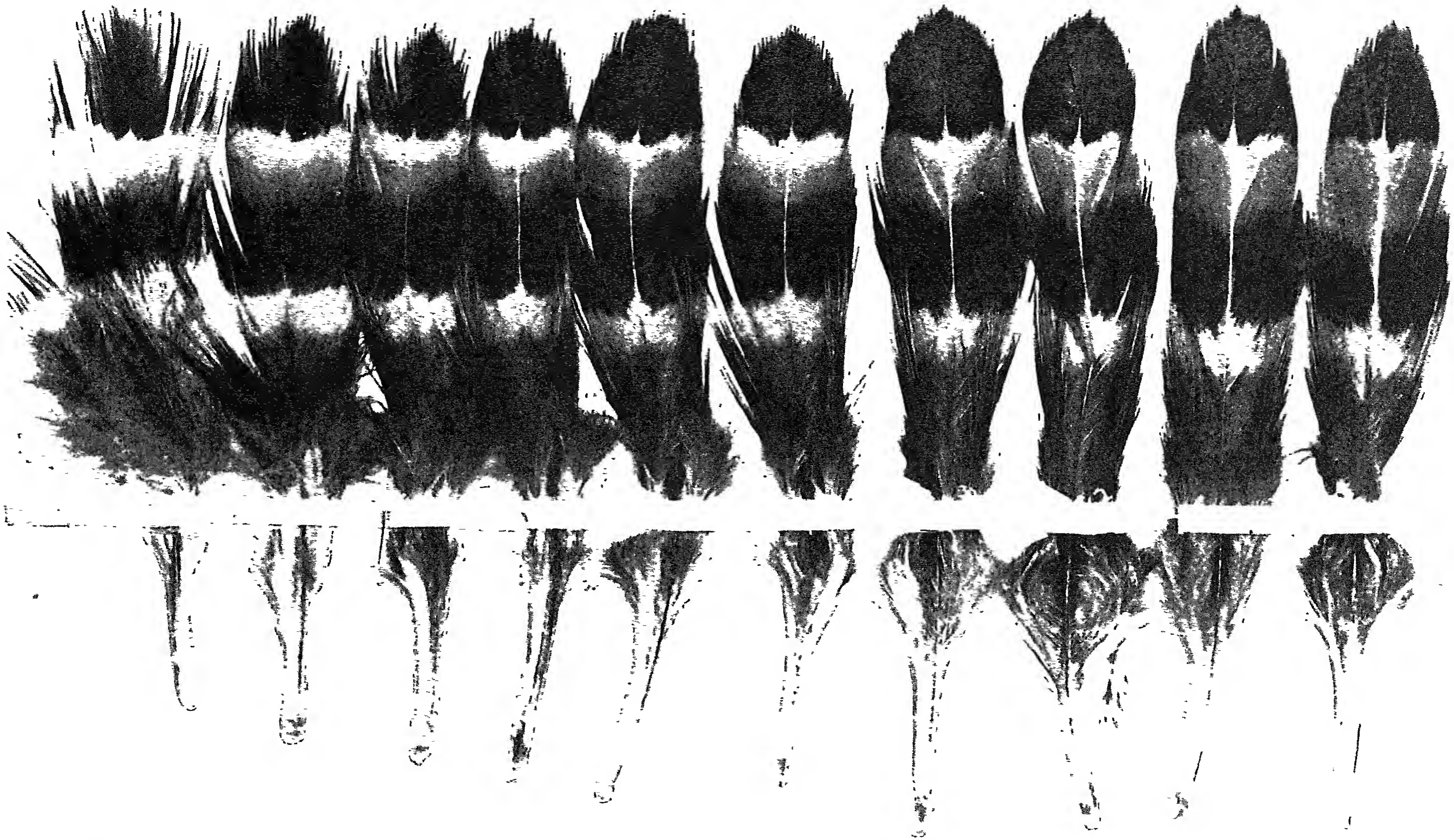
TABLE X—ANALYSIS OF 1ST AND 2ND RESPONSES INDEPENDENTLY (MEDIAN TO LATERAL RESPONSE)

	Young castrates			Mature castrates		
	Increased	No change	Decreased	Increased	No change	Decreased
Injection 1	27	14	0	18	1	0
Injection 2	2	16	23	11	6	2

The mature castrates give a rather different distribution in that more than half of the birds (11) not only exhibit an increase of the first band but also a corresponding increase of the second, fig. 12 an effect that was most rarely observed in the young group. The second largest group (6) although giving increased pigmentation change laterally at the first injection showed no tendency to increased width at the second.

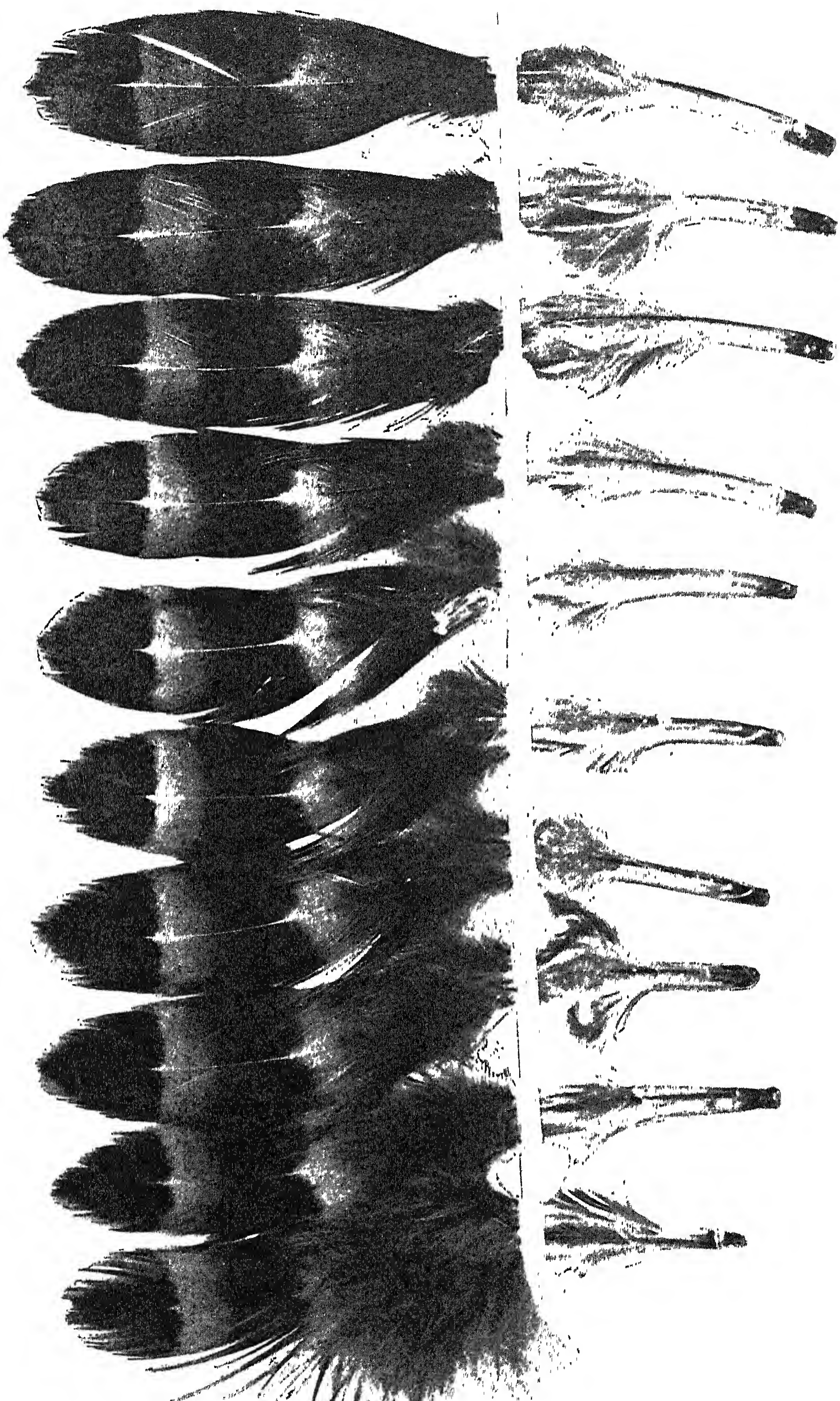
From these figures it may be postulated that the typical response to the stimulus of oestrone is, in the young castrates, modified in the median lateral direction, the first injection tending to give *progressive increases* in the width of the pigment band developed, while the second leads to a *progressive decrease* if any change occurs. In the old birds, however, while the majority respond in a similar manner to the young group at the

FIGS. 10–13—Show single transverse rows of feathers from the left breast of individual birds illustrating medio-lateral (left to right) change in response



YOUNG CASTRATES

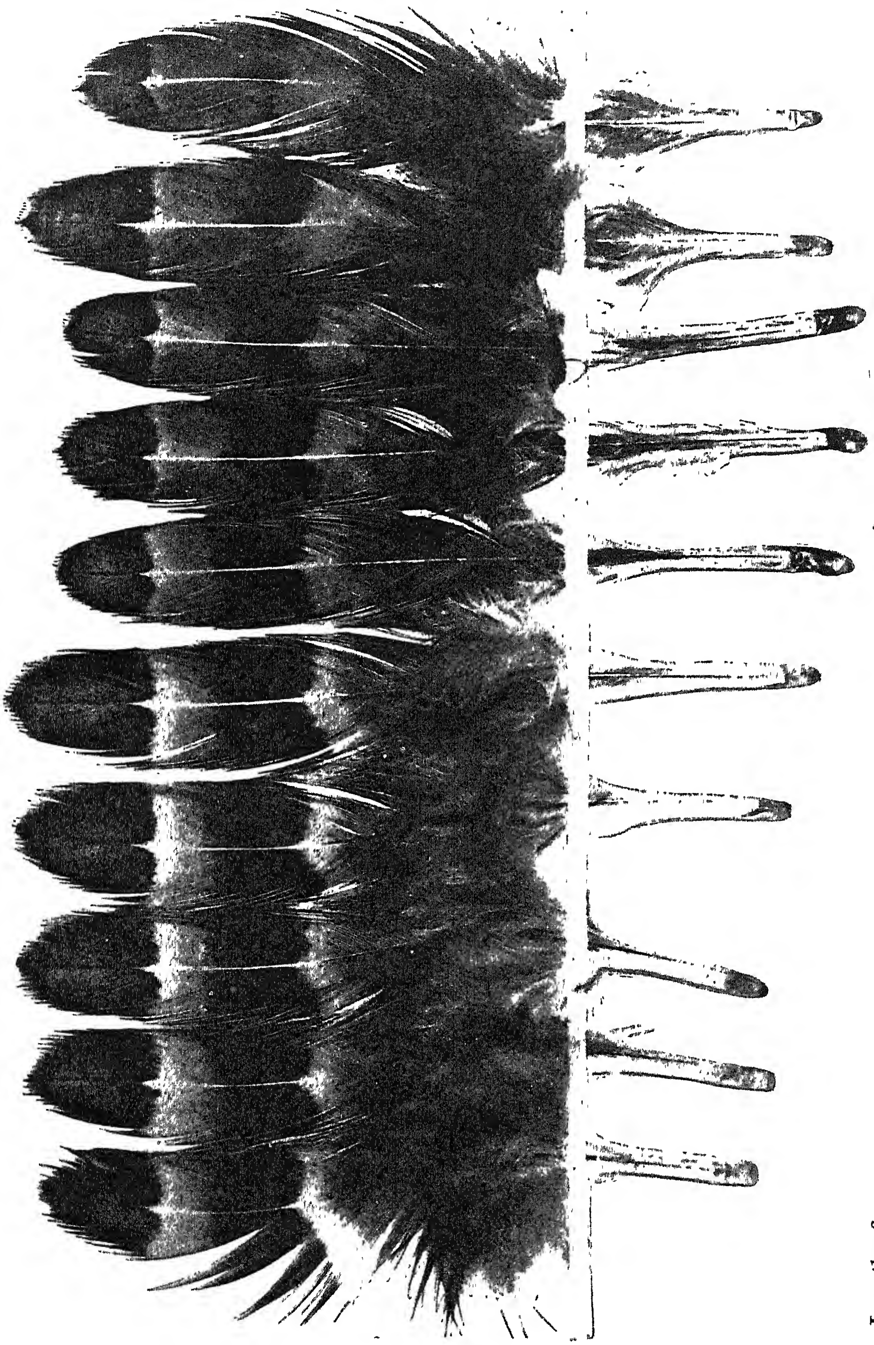
FIG. 10—Increasing response laterally in 1st bar, uniform 2nd



Length of feathers in mm 73 78 78 80 80 82 83 87 89 87

YOUNG CASTRATES

FIG. 11—Increasing 1st bar, decreasing 2nd



MATURE CASTRATES

FIG. 12—Increase laterally in both bars. It will be noted that in this bird the feathers have not all begun to regenerate simultaneously after plucking

MATURE CASTRATES

FIG. 13—One of the best responses to oestrone stimulation.

first injection, at the second there is a definite tendency to *increased* width in the pigment band. These points are made clearer by reference to Table X where the effects of the two injection series are considered separately.

Discussion

It has been shown that the response of the individual bird to the stimulus of oestrone is independent of the season of the year at which the injections were made, is likewise independent of the body weight within the limits provided by the birds used, but is markedly affected by age. Since different individuals in the population showed fairly constant and varied susceptibilities to the action of the hormone throughout a series of tests, it suggested that the general nature of the response may be controlled by genetic differences occurring in a random population. Apart from this there were very definite differences not only in individual feathers but also in a series of feathers plucked in a single row from a particular area of a bird.

It is necessary now to attempt an interpretation of the results obtained in the light of previous experimental experience. Referring to the experimental modification of feather pattern in the Brown Leghorn by the female hormone Lillie and Juhn (1932) state " As in all developmental systems, the range of physiological control is limited by the genetic organization of the species and breed. But a second limiting factor, equally decisive, also exists in the fowl in the qualities of the various feather tracts, which constitute a mosaic of self-determining feather types. It thus becomes necessary to specify not only the species and breed dealt with but also the feather tract, and in some cases *even the part of the tract*."

The fact that all feather tracts do not respond equally to the stimulus of a given quantity of hormone was originally demonstrated experimentally by Juhn and Gustavson (1930). It was ascertained by them that feathers having a more rapid growth rate required definitely higher concentrations of the hormone for the appearance of the female effect than feathers having a slower growth rate. From this the conclusion was reached by Lillie and Juhn (1932), that the female hormone concentration required for the induction of female pigmentation and feather structure in the capon is directly proportional to the rate of growth and differentiation of the male feathers in different regions.

Before entering into a discussion of the later work and the applicability of this principle of the relation between growth rate and response to the data considered here, it is necessary to determine whether the principle

of genetic differences in individuals alone would account satisfactorily for the varied types of response obtained in these experiments. The variable response associated positively with certain age groups offers no real barrier to the use of such a genetical hypothesis since a timing mechanism associated with gene action is not a rare phenomenon. The greatest objection to a purely genetic theory of control of response to the hormone lies in the fact that although the majority of the birds behaved in a similar fashion with succeeding treatments many at least show different forms of response to the same stimulus at different times. As will be seen, however, this is the same objection that can be made to the hypothesis which seeks to correlate amounts of reaction with differential growth rates.

In 1931 Juhn, Faulkner, and Gustavson extended their studies to include measurements of the growth of feathers from different body regions in a normal Brown Leghorn male and female as well as that of castrated males. Besides differences related to sex they were able to demonstrate measurable differences in the growth rate of the feathers from the different areas on the bird, as well as growth gradients in the feathers from the same areas. For instance, although the growth rate in all areas of the female was similar, in the male and castrated male it was found that growth in the anterior and posterior breast exceeded that of the other regions, namely, back and saddle. The change of the feathers to female type, by injection of oestrone to the capon, is accompanied by a change in the growth rate of the feathers in the various regions, to that characteristic of the normal female. From experiments on the production of two female bars in the feathers of the castrates it was suggested that the width of the female bar is a measure of the length of time that the concentration of the hormone in the blood remained above the threshold for that particular feather; it therefore follows that the width of the bars in the various plumage regions (and in feathers from the same region) is proportional to the growth rate.

The first of our problems to be accommodated to this hypothesis of the relation between reaction and growth rate concerns the variable response obtained in an apico-basal direction in individual feathers.

In a recent paper by Montalenti (1934) it is suggested that the growth rate along the axis of a feather is constant, and Juhn, Faulkner, and Gustavson (1931) state that, "following an initial period when the measurements were variable due to the presence of the feather sheath, which was later sloughed off, the daily increase in length of the feathers proceeded at a remarkably uniform rate." If this were so then it is to be expected that a similar stimulus should produce an identical response

at all levels of the growing feather, but as has been shown, birds that behave in this manner are rare exceptions. In the majority the reaction to the first injection measurably exceeds that at the second, a fact which makes it necessary to postulate, if the hypothesis between growth rate and response is to apply, that at different levels in the feather, differential growth rates must occur, the differences being of such an order that a differential threshold of response to the hormone is produced. With the threshold of response directly proportional to the growth rate it is obvious that the latter must increase in an apico-basal direction along the axis of the feather to account for the most general type of reaction found in these experiments. Contrary to their statement regarding the uniformity at which axial growth proceeds, the measurements of daily increases of feather length in male and female, reported by Juhn, Faulkner, and Gustavson, for a period of 24 days from plucking—the time covered by these experiments—appear to yield evidence of an apico-basal acceleration in growth rate. This is made clearer by grouping their observations on feathers of the anterior breast in four-day periods, Table XI, has been extracted from their data. While this assists in accommodating the typical reactions obtained to the hypothesis under discussion, there remain the atypical reactions, and it is unfortunate that sufficient data are not available to determine whether characteristic variations, of what may be considered the normal axial rhythm of growth rate, are to be found in a general population.

TABLE XI

Days from plucking	Average increase in feather length ♂ anterior breast in mm	Average increase in feather length ♀ anterior breast in mm
12–16	6·4	6·1
16–20	8·9	7·8
20–24	9·3	8·5

Assuming a uniform genetic control of the axial growth gradients in the feather, then the variable responses obtained would have to be interpreted as indicating that various physiological factors may act to modify, eliminate, or reverse the characteristic acceleration of growth. The fact that mature birds, on the whole, gave much better responses than the birds of the young group may be taken to imply that the former possess a much reduced level of growth, although differences in the rate, sufficient to produce different thresholds apico-basally, also exist. On the other hand, the possibility that more than one genetical factor govern-

ing the rate of growth of the feathers may exist in this stock of birds has to be taken into account, but the fact that variation in the response of an individual to a standard dose of oestrone has been demonstrated still makes inevitable the participation of a physiological factor in the process.

Although variations in response in an apico-basal direction along the axis of a single feather in individual birds may be susceptible to interpretation by means of an hypothesis relating the extent of the reaction to the growth rate of the feather at the time of injection, it is doubtful whether the modification of the response, when the median to lateral position of a feather in a particular row of breast feathers is considered, can be accommodated by this hypothesis.

Juhn, Faulkner, and Gustavson (1931) found that in the male, female, and castrate, the feathers of the breast near the median ventral line grow more slowly than the more lateral feathers, forming a graded series. This is evident from the length of the feathers developing when a single row of breast feathers is regenerated after simultaneous plucking, figs. 10–13. It will be remembered, however, that in the majority of birds the extent of the first reaction *increased* in the feathers passing laterally from the median ventral line, whereas on the hypothesis of a positive correlation between rate of feather growth and threshold of response to the stimulus of the hormone, it is to be expected that as the growth rate increases the width of the colour band should *decrease*.

If, however, the greater width of the bar laterally is taken to indicate a lower threshold of response with a correspondingly lowered growth rate in this particular region of the feather, then in all such cases, the growth rate basally must be much increased since the final length of the feather is to exceed that of those more medially situated (in the same time period). Thus it might be expected that feathers showing increase in the first bar laterally would show a complementary decrease in the width of the second pigment bar: roughly one-third of the young birds exhibited this type of behaviour while an equal number show an increase in the first without significant change in the second pigment bar.

While both of these findings may be considered to fit an hypothesis of a variable axial growth gradient in the feathers from centre to side of the breast, it is not evident that the reaction commences significantly earlier laterally, as would be expected were the threshold of response to the stimulus to be lowered to any considerable extent, figs. 10–13.

Again, in the mature group, where the largest number of birds showed lateral increases in both bars, a lowered growth rate for a considerably greater length of the vane would have to be postulated, and this despite

the fact that here also the outside feathers appear to have the most rapid average growth.

A striking feature of the reaction in this class is the encroachment of the broadening red pigment bars on the intervening melanic region, sometimes even to the point of its disappearance laterally, while medially it is still well marked: superficially at least, this appearance is in accordance with the idea of a lowered threshold of response associated with a lowered growth rate.

So far then it has been possible to accommodate our results to Lillie and Juhn's hypothesis but only by assuming that, contrary to their experience, changing axial growth gradients in the feather must exist. In the absence of sufficient evidence on this point from normal birds it may be considered whether measurements of the various pigment bars on the feathers would offer assistance in clarifying the position. In commencing an examination of this kind it must be remembered that Juhn, Faulkner, and Gustavson have found that the typical pigmentary reaction of the breast feather to oestrone is accompanied by a lowering of the growth rate to that typical of homologous feathers in the female, so that feathers exhibiting wide pigment bands will be shortened to a relatively greater extent than those in which the effect is not so well marked. Allowing for this however, since the proportional lengths of the feathers remain much the same, it is to be expected that where a marked lateral increase in the red pigment band occurs, the length of the feather below the red should be proportionally much longer in relation to the median feathers than occurs in feathers showing a less well marked medio-lateral divergence. Secondly, the length of the feather from apex to the onset of the second bar should show a decrease laterally in relation to the median feathers which is greater in the group where both pigment bars show a progressive increase than in those where the width of the bars is uniform or where the second decreases, the modification of the growth rate, to that of the female, tending to accentuate this difference.

Measurements relative to the first suggestion did not give the expected results, for equally large differences occur, basally, between the median and lateral feathers, where the bars are narrow and uniform as well as where they are wide and show well marked increases laterally.

Neither does the second set of measurements bear out the point under review, for, in the majority of birds, irrespective of their reaction type, a slight increase in the apical length occurs laterally.

It might be argued that, in the second test applied, the issue is clouded by the fact that as the threshold of response decreases, the time the effect

takes to appear in the feather (the reaction rate) becomes longer; this variable can be excluded or balanced, however, if the apical measurement is taken as the width between the points of appearance of the two red bars. In this case again the results do not support the hypothesis, for in all the birds this distance shows little or no medio-lateral change, a circumstance which would be impossible if there were a direct relation between threshold of response and growth rate.

The results obtained, then, indicate that, although a theory of correspondence between growth rate and reaction may fit the majority of the responses obtained when they are considered in relation to, as yet hypothetical, apico-basal growth gradients in the feather, it cannot be said to offer a satisfactory explanation of the variation in amount of reaction to an identical stimulus when similar zones are considered in a medio-lateral direction on the bird.

That the observations in these experiments disclose an unexpectedly wide range of variation when compared with that found by the Chicago workers may be correlated with the relatively large population of birds employed. (In their publications they refer to the fact that the individual birds show some variation in the degree of response.) The technical procedure varied somewhat also; for example, (1) the solvent used in these experiments was alcohol instead of olive oil, and in the latter the absorption rate of oestrone into the general circulation is considerably slower (Deanesly and Parkes, 1933), so that when this is used, some delay in reaching the threshold of response in any given feather may result. It may also affect the length of time at which the hormone in the blood remains above the threshold level. (2) All injections in this study were made deep into the pectoral muscles of the breast whereas in the previous experiments, subcutaneous injections alone were employed, and it is difficult to appreciate the extent to which this variable may influence the reaction in the absence of further experimentation. In the latter connection, however, it has been possible to show that subcutaneous or intradermal injections of minute doses of oestrone readily produce localized effects in feathers, and further that there is a definite positive correlation between the amount of response produced and the distance of the feather from the point of injection.* It is probable then, that where ordinary doses are used the concentration of the hormone, in the region of the subcutaneous injection, is considerably above that in the general circulation, and that in the reactions resulting, much would depend on the proximity, or otherwise of the feathers examined, to the site of injection.

* See the succeeding paper, Part II.

From the occurrence of these localized effects a final point may be made. A direct action can be established between oestrone and the pigmentation change in male feathers, but the physiological action of oestrone, resulting in a modified growth rate, is not so susceptible to demonstration; in the latter reaction the hormone may yet be proved to act, not directly on the feather, but through an intermediary, in which case a difference between the peripheral concentration of the oestrone and that in the blood may provide a means of testing an hypothesis that depends solely on the apparent relation between the growth rate of feathers and the amount of pigmentary change induced.

The expenses of this work were defrayed by a grant from the Medical Research Council.

Summary

A study has been made of the variation in the response of breast feathers of Brown Leghorn capons to a standard dose of oestrone.

The variations obtained in four tests, covering the period of one year were examined, their frequency classified, and the possible influence of body weight, season of year, and age of birds on the magnitude of the responses considered. Because persistent differences in the magnitude of the response in individual birds occurred, it was concluded that genetic factors were involved.

Within the limits of the material examined, no significant variation due to body weight, or to season could be demonstrated.

A marked effect of age on the response of the younger of two groups of birds studied was shown. The age of the birds in the immature group varied from 7·5 to 8·5 months, those of the mature group from 1·5 to 5·5 years. The average amount of response of the immature birds was considerably below that of mature ones.

Differential responses in the individual were disclosed; these took the form of changes in the amount of reaction (*a*) in an apico-basal direction along the axis of the feather, and (*b*) in a medio-lateral direction in the feather tract. Antero-posterior relations in the breast tract were not studied.

An examination of the whole range and trend of variability encountered, in the light of the results of previous work in this field, showed that while it was necessary to postulate that both genetic and physiological factors were involved in their production, the nature of the latter influence could not be positively determined, and that the hypothesis seeking to correlate

growth rate in the feather with threshold of response to the hormone, was insufficient to accommodate all the modifications obtained.

References

Cook, J. W., Dodds, E. C., and Greenwood, A. W. (1934). 'Proc. Roy. Soc.,' B, vol. **114**, p. 286.

Deanesly, R., and Parkes, A. S. (1933). 'J. Physiol.,' vol. **78**, p. 155.

Juhn, M., and Gustavson, R. G. (1930). 'J. Exp. Zoöl.,' vol. **56**, No. 1, p. 31.

Juhn, M., Faulkner, H. F., and Gustavson, R. G. (1931). 'J. Exp. Zoöl.,' vol. **58**, p. 69.

Lillie, F. R., and Juhn, M. (1932). 'Physiol. Zoöl.,' vol. **5**, p. 124.

Montalenti, G. (1934). 'J. Exp. Zoöl.,' vol. **69**, No. 2, p. 269.

---

612 . 018 . 2 : 612 . 799 . 7

Variation in Plumage Response of Brown Leghorn Capons to Oestrone

II—Intradermal Injection

By A. W. Greenwood and J. S. S. Blyth

(Institute of Animal Genetics, University of Edinburgh)

(Communicated by A. S. Parkes, F.R.S.—Received, March 2, 1935)

[Plates 4–7]

Introduction

In the first part of this paper, reference was made to the development of a new technique for obtaining the induction of female pigmentation in the breast feather of Brown Leghorn capons by means of oestrone. The intricate and lengthy process necessary for the production of this substance makes it desirable that economy in its use be practised. Accordingly our primary intention was to investigate the possibility of influencing individual feathers, and while this was not realized, it was found that a local effect in a restricted area could be obtained with relatively minute doses of oestrone. The chances of either of these conditions occurring could not be foretold since it was not known whether female hormone

acted directly on the feather, through an intermediary (*e.g.*, the thyroid gland), or even had to undergo chemical change before it could prove effective.

Technique

The first attempts to obtain a localized effect on the feathers were made in two ways: (1) intradermal injections at the base of a feather in the manner described below, and (2). injections into the feather pulp itself. The second method, however, was discarded as unsatisfactory since, although it yielded positive effects the pulp was found to be extremely dense and it was impossible to introduce a measurable quantity of fluid into the feather with the apparatus available. A further objection to this technique was that the feather was frequently damaged, and either died or ceased to grow.

The intradermal method held out more promise in that, even if one or more feathers, immediately adjacent to the point of injection, were damaged or destroyed, figs. 3, 4, 6, and 8, Plate 4, the rest of the feathers in the surrounding region were not injured, and where a sufficient amount of oestrone had been injected, showed the typical pigmentary changes. Later, subcutaneous injections were also used in this series of experiments.

As in previous experiments, feathers were plucked prior to the injections, but in this case three adjacent rows were denuded, sometimes on both sides of the breast. A diagrammatic illustration of the spatial relation of the feathers in the breast tract is shown in Plate 7, feathers in the same and adjacent rows overlap but have been shown completely separated in the diagram for the sake of clarity. In order that the behaviour of individual feathers might be studied in relation to their orientation they were numbered serially, beginning with the most ventral member of the anterior row, counting outwards along each row, and ending with the most lateral of the posterior series. This made easy the determination of the exact position of any feather plucked from the area irrespective of the time of removal. Further, some such method of identifying individual feathers was necessay in order to compare and discuss the results obtained here with those of Juhn and Fraps (1934).

In all the experimental birds an attempt was made to denude an identical feather region, the anterior row plucked being that nearest to and running parallel with the furcula.

The injections, which were of the order of 0·05 to 0·15 cc, were given by means of a small tuberculin syringe, graduated to 0·01 cc, with a correspondingly fine needle. In the intradermal injections the fluid

was introduced between the layers of the skin, and could be seen extending it in the form of a bubble. It was found that about 0·10 cc was about the maximum amount of fluid which could be administered in this way without the occurrence of leakage. It was not possible to observe similarly the position taken up by the material when injected subcutaneously, since the subdermal tissues are very loose and did not exert any obvious restriction on the small quantities of fluid introduced among them.

Material

The birds, 15 in number, which participated in this experimental series, were some of the Brown Leghorn capons whose feather response to oestrone administration had been under observation previously in a study designed to examine the variability of reaction to intramuscular injections of a standard dose, *see* Part I. The oestrone was also taken from the stock provided through the generosity of M. André Girard, of Paris, and was used in the same concentration as previously, *i.e.*, the solution injected contained 0·25 mg/cc of crystalline oestrone in 60% alcohol. Besides this alcoholic solution, which might be regarded as our control substance, a few tests were made in which the oestrone was dissolved in sesame oil.

Apart from a number of preliminary tests, which it would serve no useful purpose to discuss here, the data relevant to these experiments are given in Table I.

Results

As was to be expected neither bird receiving the injection of oestrone intramuscularly gave a positive response, but all the birds in which intradermal or subcutaneous injections were used showed, in the regenerating feathers in the plucked areas, a change in the type of pigmentation from black to red, the amount of the change induced being approximately proportional to the dosage employed. In Plate 4 a schematic representation of the feather tracts is given for those birds in which a positive response was obtained, showing the site of injection ×, and the number of feathers in the plucked area which were affected, ●, by the treatment. Feather follicles failing to regenerate new feathers either as a result of damage following the injection or from causes unconnected with it are omitted from the diagram.

In those birds receiving the lowest dosage the extent of the medio-lateral effect is seen to be within the limits of the plucked area; the antero-posterior extension has not been determined since, in all birds,

the region plucked consisted of three transverse rows only. Where heavier doses of oestrone were employed, it will be seen in the majority of birds that the effect covers the whole area and probably extends beyond it. This was shown to be so in No. E 712, figs. 8 and 9, Plate 4, which was plucked on both sides of the breast before injection, and after an intradermal injection of 0·025 mg of oestrone in the right denuded area, fig. 8, exhibited a marked effect on that side, and a faint but definite effect in the regenerating feathers of the left side, fig. 9, indicating that the dosage had been large enough to induce a minimal general effect in the bird. A difference in the number of feathers affected by the same dose of

TABLE I

No. of bird	Dose in cc	Amount of oestrone in mg	Solvent	Method of injection	Result	*Cf.* Plate 4 Fig.
E 317	0·05	0·0125	Alcohol	Intradermal	+	1
E 536	0·05	0·0125	,,	,,	+	2
E 426	0·05	0·0125	,,	,,	+	3
E 102	0·05	0·0125	,,	,,	+	4
E 738	0·05	0·0125	,,	,,	+	5
E 585	0·05	0·0125	,,	,,	+	6
E 230	0·05	0·0125	,,	,,	+	7
E 403	0·05	0·0125	,,	Intramuscular	–	—
E 679	0·05	0·0125	,,	,,	–	—
E 712	0·10	0·0250	,,	Intradermal	++	8, 9
E 187	0·15	0·0375	,,	Subcutaneous	++	10
E 179	0·15	0·0375	,,	,,	++	11
E 327	0·10	0·0250	Sesame oil	Intradermal	++	12
E 144	0·05	0·1000	,,	,,	++	13
E 398	0·05	0·1000	,,	Subcutaneous	++	14

hormone in different birds may be remarked, figs. 1–7, Plate 4, and this is in line with the results obtained from the previous study in which characteristic individual reactions were observed.

Both intradermal and subcutaneous methods gave, with either alcohol or sesame oil as a solvent for the oestrone, satisfactory responses, but the possible existence of a difference in the efficacy of the techniques employed cannot be demonstrated from the present material.

The distribution of the red pigment which appeared in the growing feathers took a characteristic form, being greatest in those in the immediate vicinity of the point of injection and waning as feathers further from it were examined, Plates 5 and 6.

Peculiar also to this type of hormone administration is the number of feathers affected asymmetrically. This is illustrated by photographs of the feathers in Plates 5 and 6. In the three denuded rows, it was found that feathers exhibiting an asymmetrical distribution of the red pigment were in the main, arranged in a definite manner with the more affected side next the feather row in which the injection was made, irrespective of whether they were near or far from the point of hormone administration. In a number of feathers the asymmetry produced took the extreme form of hemilateral bars of red pigment, fig. 26, Plate 6, but in the majority both sides of the feather vane showed the typical pigmentary change. From close examination of those feathers in which the response was not particularly well marked it was possible to determine whether or not a distinct preponderance of pigment on one or other side of the vane was present.

TABLE II

	Row 1			Row 2			Row 3		Total	
Feather number	R	L	Feather number	R	L	Feather number	R	L	R	L
1	—	—	10	—	—	19	—	1	—	1
2	4	—	11	—	3	20	—	—	4	3
3	3	1	12	2	2	21	—	2	5	5
4	2	—	13	2	1	22	—	2	4	3
5	—	—	14	1	—	23	—	1	1	1
6	2	—	15	1	—	24	—		3	—
7	—	—	16	—	—	25	1	—	1	—
8	—	—	17	—	—	26	—	1	—	1
9	—	—	18	—	1	27	—	—	—	1
Total......	11	1		6	7		1	7	18	15

A classification of feathers in which an asymmetrical pigment distribution occurred has been made relative to their position in the particular region studied, Tables II and III, those feathers in which an equitable distribution was found on both sides of the vane have not been included. From Table II, the data for which were derived from macroscopical observations on the feathers, it will at once be obvious that there is a characteristic distribution of feathers showing one or other type of asymmetry, feathers from the first row exhibiting almost exclusively a greater amount of red pigment on the right half of the feather vane while in the third row this phenomenon is reversed. On the other hand both types of asymmetry are evenly distributed in the central row of feathers. If a rather more extensive classification is employed based on a microscopical analysis of the affected feathers it will be seen that the same

relationship persists, Table III. The significance of these findings will be discussed in detail later, but the relation between the distribution of types of asymmetry and the point of hormone injection should be emphasized; in every bird used in these experiments the injection was made at different levels between the feather follicles of the *second* plucked row, *see* Plate 4.

TABLE III

	Row 1		Row 2		Row 3		Total	
	R*	L	R	L	R	L	R	L
Total............	29	5	19	17	4	24	52	46

* In both Table II and III, R and L signify that the greater amount of red pigment was deposited in right and left half of the feather vane respectively.

DISCUSSION

The fact that it is possible to obtain a response to the administration of oestrone in a particular part of the plumage has already been shown by Juhn and Gustavson (1930) who found that, with suitable doses, female characteristics could be produced in the saddle feathers of the Brown Leghorn capon without affecting those of the breast region. Further, they demonstrated that the appearance of the salmon colour of the female could be induced in the anterior feathers of the breast region without influencing those more posteriorly situated. They later showed (1931) that the different thresholds of response to the hormone on the part of feathers found in these various regions could be correlated with their respective growth rates, and from these findings the hypothesis of a direct relation between threshold of response and the growth rate was enunciated (Lillie and Juhn, 1932, and Part I).

In addition to an antero-posterior growth gradient in the feathers of the breast region, Juhn, Faulkner, and Gustavson (1931) also found that feathers near the median ventral line grow more slowly than the more lateral ones so that a medio-lateral growth gradient also exists. But if the growth rate increases in a lateral direction it will be seen that the results obtained in these experiments were of a kind not encountered by these authors, and are incompatible with the hypothesis of a direct relation between growth rate and amount of response, for the reaction obtained in our cases is always greatest around the point of injection of the hormone and wanes in both directions along the tranverse rows, Plates 5 and 6, irrespective of the fact that the growth rate is increasing in one direction and decreasing in the other.

In a more recent publication Juhn and Fraps (1934) have stated that "when the mark due to the hormone was restricted to one vane-half in the definitive breast feather, other feathers were occasionally found in the same tract bearing a similar mark on the opposite vane-half." They demonstrated that with regard to this asymmetrical distribution of pigment there is a true reversal of symmetry within each breast tract, the feathers on the inner, ventral part of the breast showing the effect on one side of the vane and those on the lateral part on the other, the sixth antero-posterior row from the median line being about the point of reversal. While they deduce that this behaviour is correlated with differential growth rates, which alter in their relation to one another in a medio-lateral direction, in the two vane halves of the feather, they admit some difficulties in the way of this conclusion. Be that as it may, it is clear that the responses obtained in the present experiments are of a different order, and are in line neither with their observations nor their conclusions. The asymmetrical patterns developed do not, in these experiments, take their arrangement from an antero-posterior line, but depend for their distribution on a movable focus, the point of injection of the hormone.

It is evident then that neither the distribution of the red pigment among the feathers of the tract nor the position it takes up in the individual feather is what would be expected from previous observations, nor are they susceptible to the same explanation. In the first reaction, it would seem rather that the oestrone acts directly on the feather follicle itself, and that the decreasing effect is brought about by the gradual diffusion of the hormone through the tissues, with the consequent lowering of its concentration as it spreads until finally it is too weak to influence feather characterization. This reaction would indicate the necessity of adopting a cautious attitude when comparing responses elicited by subcutaneous injections, for the effect produced might vary considerably according as the feathers examined were derived from a point near or far from the site of injection.

In considering how the oestrone in these experiments reaches the growing feather in order to exert its characteristic action, two possibilities have to be taken into account: (1) either it is carried there by the blood stream, or (2) it makes its way through the intervening cells and tissues by osmosis. In view of the mode of distribution of asymmetrical pigmentary changes in the feathers (which from the foregoing discussion cannot be attributed to their inherent potentialities) it may not be readily accepted, in the absence of definite evidence, that the vascular system is the means of transport; for this necessitates the postulation that each vane half of

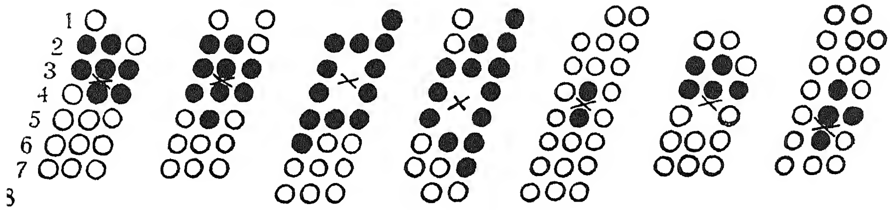


FIG. 1 FIG. 2 FIG. 3 FIG. 4 FIG. 5 FIG. 6 FIG. 7

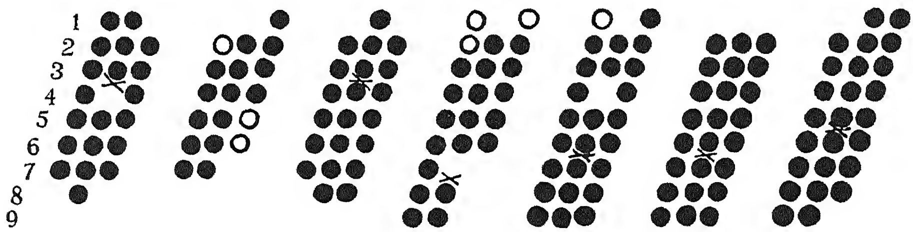
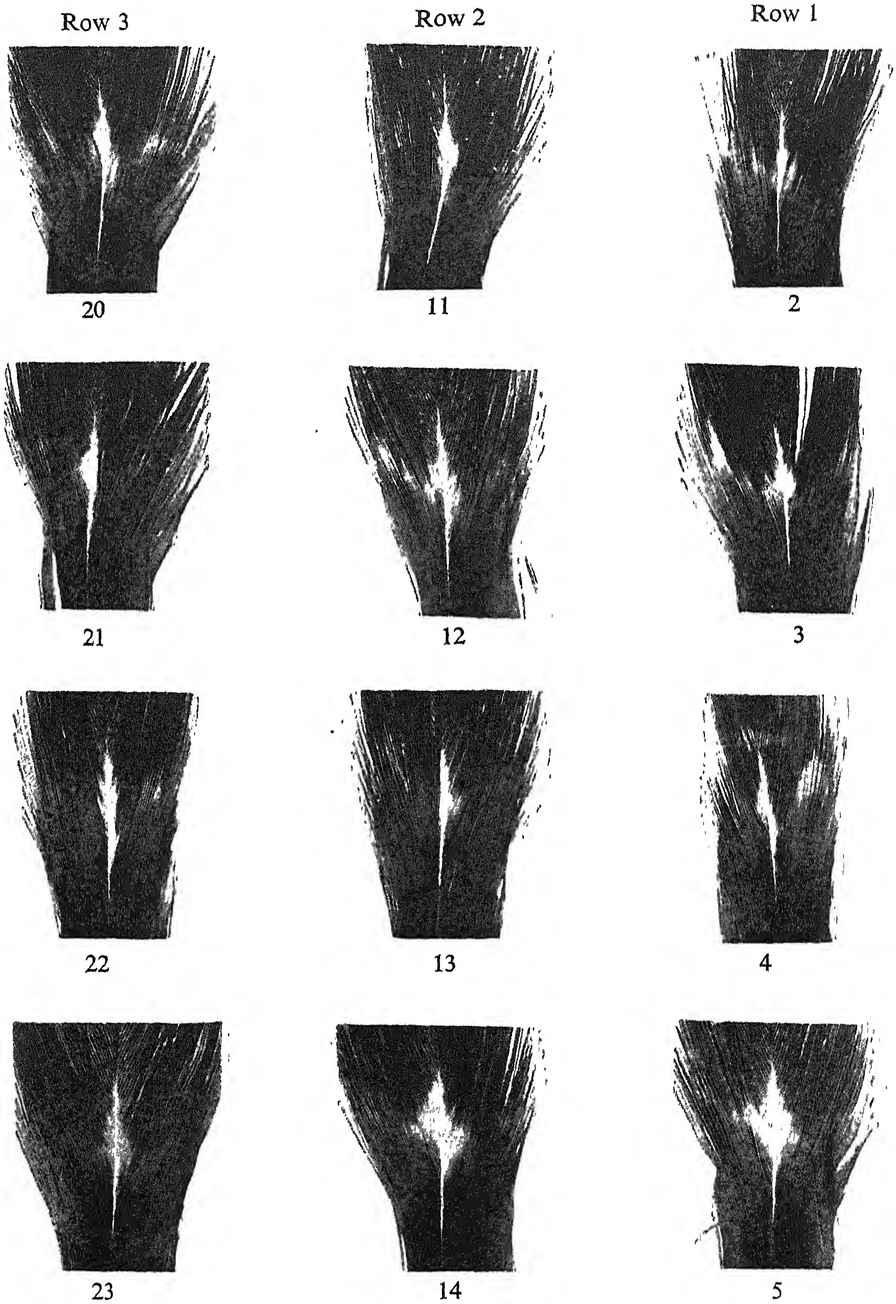


FIG. 8 FIG. 9 FIG. 10 FIG. 11 FIG. 12 FIG. 13 FIG. 14

FIGS. 1–14—Diagrams illustrating localized reaction of feathers to oestrone (*see* Table I for dosage). ●, affected feathers; ○, feathers which regenerate successfully after plucking but showed no response to the injection.



FIGS. 2–9, 11–18, 20–27—Bird No. 144. Intradermal injection (right side) of 0·025 mg oestrone in sesame oil. The feathers are photographed from the back so that the orientation of the rows is reversed, *cf.* Plate 7. Note (1) the waning effect of the hor-

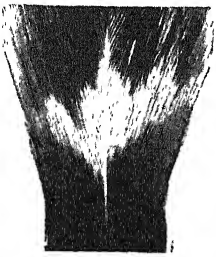
Row 3 Row 2 Row 1

24

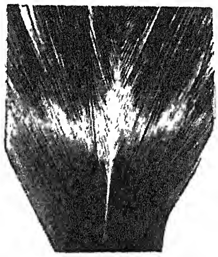
15

6

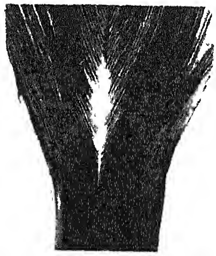
X



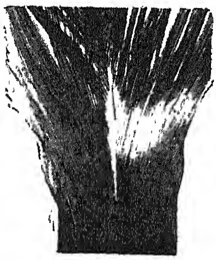
25



16



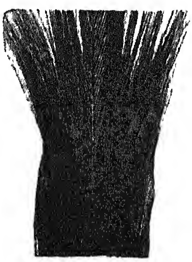
7



26

17

8



27

18

9

mone in feathers progressively further from the point of injection X, and (2) the marked asymmetry of pigment deposition particularly in figs. 3, 6, 12, 13, and 26.

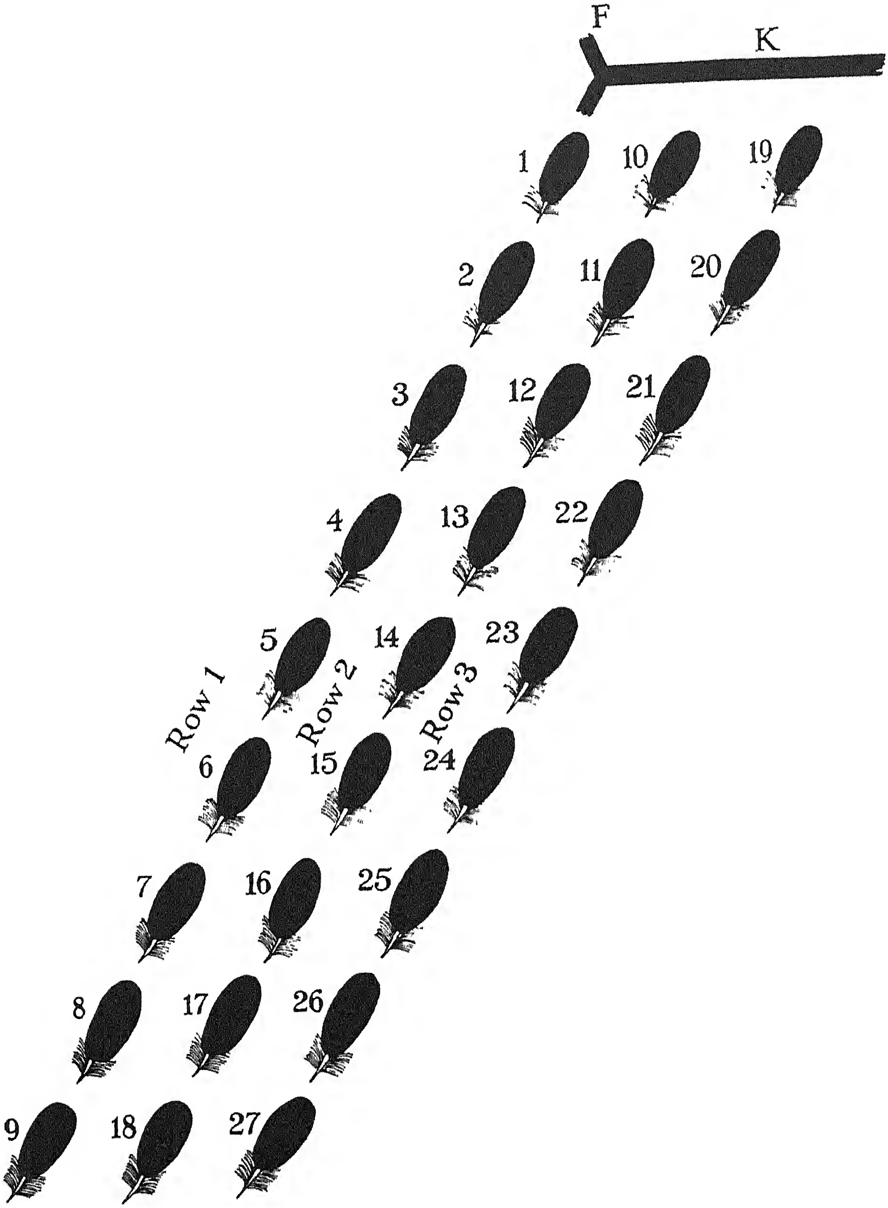


Diagram showing the orientation of the three plucked rows on the right breast of the bird in relation to the keel, K, and the furcula, F. Feathers numbered as described in the text. (Actually in life the full-grown feathers curve posteriorly and overlap in both directions.) Where reference is made in the text to the right and left halves of feathers, they are always used in relation to this diagram. Feathers on the left side of the body are mirror images of those illustrated.

the feather has an independent blood supply. On the other hand, the effects obtained do lend some support to the suggestion of a direct diffusion of the hormone through the tissues, since the grades of effect tend to run parallel to the transverse rows of feathers in the breast. This would agree with the fact that the tissues are looser between the feather rows, and that the denser cell structure of the feather follicles themselves and their relative proximity to one another, offer some hindrance to the passage of the hormone between them.

The question of how asymmetrical patterns themselves develop is perhaps the most intriguing one. In the course of this study we have recognized two different modes of their distribution among feathers in a selected area (breast), namely, that obtained by previous workers following subcutaneous injections of considerable amounts of hormone (Juhn and Fraps, 1934) and that found in our own experiments with intradermal and subcutaneous injections of small doses of oestrone. Reference to the reactions obtained in the experiments with relatively large doses described in Part I provides further data on this subject: out of 64 capons used in these tests, 15 exhibited half bars of red pigment on the most lateral of the plucked breast feathers, and in all birds these were on the median side of the feather, *i.e.*, in feathers from the left side of the breast it occurred on the right side of the vane, and in feathers from the right breast on the left vane. In the more centrally placed breast feathers asymmetrical patterns were not found, except where the bars were very narrow and only faintly delineated, and in these conditions they were not consistently associated with either vane half.

In seeking an explanation for the appearance of asymmetrical patterns, the two main possible causes may be examined: (1) that they result from a differential reactivity in the two sides of the feather, or (2) from a differential stimulation. That the two vane halves can behave independently is clearly seen in the wing primaries and secondaries of the Brown Leghorn; in the latter marked differences in colour and structure may be observed on the two sides of the rachis, while a close examination of the former shows that proximally some of them widen out on one side without a coincident alteration appearing in the other vane half. (The widening is actually due to a shortening of the barbs and an increase in their number.) Such observations make it evident that the two vane halves may be considered as separate entities, but one cannot infer that they are nourished independently for the asymmetries of colour and structure which occur naturally probably have their origin in genetic differences and are not due to unequal stimulation by environmental influences. Such a view may be deduced from the skin transplantation experiments

of Danforth (1929) which showed that the size of the feathers on an implant was determined by the donor of the graft and not by the host, and also from a capon in our own stock in which a patch of atypical feathers occurs in the anterior breast; these are similar to feathers from the cape region in both colour and structure.

From these considerations too it is reasonable to assume that the relation of the growth rates on the two sides of the feather, with which Juhn and Fraps associate the position of the asymmetrical patterns, is also a condition inherent in the feather so that the pattern behaviour obtained appears to fit the first hypothesis. The atypical lateral breast feathers from our intramuscularly injected series, appear at first sight to be similar to those encountered by the authors just mentioned, for the larger amount of red pigment is situated on the vane half which, according to their measurements, has the shorter barbs and therefore the lower threshold of response. The lack of coincident well-defined asymmetrical patterns on the feathers nearer the median line of the bird leads us to put forward another suggestion as to the behaviour observed.

It may be considered that the action of oestrone is to inhibit the deposition of melanin in the feathers (Greenwood and Blyth, 1929); this action is practically complete in the breast region of the female but incomplete in other parts of the body such as the back and saddle where the melanin is restricted to fine broken lines resulting in a characteristic pencilled pattern. In females in which the black pigmentation is not entirely absent from the breast it is found to be concentrated mainly in the lateral feathers: faint stippled patterns occur, becoming more marked towards the outer edge of the tract, and always heavier on the outer (right) side of the feather, *i.e.*, on the opposite side from that on which the half bar of red pigment was found in the experimental feathers. Evidence that male birds have similar potentialities has been obtained from capons bearing ovary grafts as well as from some of the birds under experimentation in which black flecking in the bars of red pigment has been noted, and it seems probable that the half bars encountered resulted from the inherent inability of the feathers in this particular region of the breast tract to react completely to the restricting action of the oestrone.

That this reaction is confined to the outer lateral feathers while changing relations in barb length of all the feathers in the tract exist (*vide* Juhn and Fraps) suggests that two phenomena are being expressed both indicating a differential reactivity on the two sides of the feather. But, on the other hand, the postulation just put forward, raises a difficulty in regard to the acceptance of the conclusions of the Chicago workers. Melanin is restricted to a much greater extent on the breast than on the back of the

female, yet according to the measurements of Juhn, Faulkner, and Gustavson (1931) the growth rates in these two areas are much the same, indicating on their hypothesis, a similar threshold of response to oestrone. Even if it is assumed that it is the basal growth rates present in the poularde, ovariectomized female, that are concerned, the theory is not improved, for it is to be expected that in the latter as in the capon, the rate of growth is slower on the back, which would result in the greater melanin restriction in that region.

The inference then is, that the two forms of female pattern discussed must arise through inherent tendencies unassociated with growth rates in the feathers, and that although the black stippling found in the breast feathers may appear to be distributed as expected from the " growth-rate-threshold " hypothesis, it is difficult to envisage that what are to all intents grades of the same patterning—pencilling in the breast and back feathers—should in one form be dependent on the growth rate and in the other unrelated to it.

Whatever the actual cause of the variations in patterning, it must be concluded that the asymmetrical modifications considered, whether they are in reality two phenomena or one, are brought about by some inherent quality of the feather. In the asymmetrical distribution of pigment resulting from the intradermal injections, however, it has been shown already that the reactions induced are not susceptible to explanation along the lines of the theory of Juhn and Fraps (1934), and it is equally clear that they cannot be accommodated by our postulation based on the asymmetry resulting from intramuscular injection. The fact that the amount of pigmentary change induced in the two vane halves of feathers bears a definite relation to the site of injection makes it impossible to relate the response to any inherent tendencies of the feathers themselves and forces us to the conclusion that a differential stimulation takes place in these experiments which is capable of overriding the normal expression of the genetical pattern factors.

The expenses of this work were defrayed by a grant from the Medical Research Council.

Summary

By means of intradermal and subcutaneous injections of relatively small amounts of oestrone, it has been found possible to induce a local effect in feathers from a restricted area on the breast of the Brown Leghorn capon. The effect produced was most marked in feathers near the point of injection and waned as those further from it were examined.

While a dosage of the order of 0·0125 mg of oestrone produced a purely local response, 0·025 mg was sufficient to induce positive responses in feathers regenerating on both sides of the body although the effect on the non-injected side was less marked. This result indicates that the action of oestrone on feather pigmentation—the replacement of black by red—is a direct one, since only in the latter case was the concentration of oestrone in the general circulation great enough to induce positive effects in the feathers at some distance from the injection point.

The red pigment bars produced were frequently unsymmetrical in the two vane halves of the feather and their shape bore a definite relation to the site of injection, the effect being greater on the side nearer to that point. The arrangement of the asymmetrical patterns suggests that the oestrone exerts its influence by diffusion through the intervening tissues and not through the general circulation.

That the type of distribution of asymmetrically marked feathers in this case differs from that obtained by previous workers, and also from that encountered in the series of intramuscular injections is discussed.

References

Danforth, C. H. (1929). 'Arch. EntwMech. Org.,' vol. **116**, p. 242.

Greenwood, A. W., and Blyth, J. S. S. (1929). 'Proc. Roy. Soc. Edin.,' vol. **49**, p. 313.

Juhn, M., and Gustavson, R. G. (1930). 'J. Exp. Zool.,' vol. **56**, p. 31.

Juhn, M., Faulkner, G. H., and Gustavson, R. G. (1931). 'J. Exp. Zool.,' vol. **58**, p. 69.

Juhn, M., and Fraps, R. M. (1934). 'Proc. Soc. Exp. Biol. & Med.,' vol. **31**, p. 1187.

Lillie, F. R., and Juhn M. (1932). 'Physiol. Zool.,' vol. **5**, p. 124.

611 . 018 . 4

Experiments on Skeletal Growth and Development *in Vitro* in Relation to the Problem of Avian Phokomelia

By Honor B. Fell, Royal Society Messel Research Fellow, and Walter Landauer

(From the Strangeways Research Laboratory, Cambridge, England, and Storrs Agricultural Experiment Station, Storrs, Connecticut, U.S.A.)

(*Communicated by R. Robison, F.R.S.—Received March* 6, 1935)

[Plates 8 and 9]

Introduction

The experiments to be reported in the following pages were suggested by observations made by one of us on the so-called Creeper fowl. Creeper chickens are characterized by a disproportionate shortness of the long bones of the extremities. Histological study has shown that Creeper chickens belong in the same category as the disproportionate dwarfism of mammals known as chondrodystrophy or achondroplasia (Landauer, 1931). The Creeper characters are inherited as a Mendelian dominant and are lethal in homozygous condition (Landauer and Dunn, 1930). Homozygous Creeper embryos generally die after about 72 hours of incubation, but in rare cases they survive beyond this stage and continue development up to nearly hatching time. These late stages of homozygous Creeper embryos exhibit striking malformations of the extremities which are known as phokomelia (Landauer, 1933). A study of the early embryonic development of homozygous Creeper embryos (Landauer, 1932) led to the conclusion that the effects of the Creeper mutation are not brought about by specific gene action on those body parts which later show deformities, but by a general retardation of body growth at a definite stage of development. This conclusion was strengthened by a detailed comparison of embryonic and post-natal bone growth in heterozygous Creeper and normal chickens (Landauer, 1934). All evidence which so far has been obtained in this work points to the conclusion that the characteristic traits of heterozygous as well as homozygous Creeper chicks are produced by an unspecific retardation of development at a time when formation of the buds of the extremities (and of the head which in homozygous embryos also shows deformities later on) are proceeding

at a particularly rapid rate, thereby causing specific disturbances in the differentiation of these parts.

It seemed to us that it should be possible to put these conclusions to an experimental test. The most promising way of approach appeared to be an attempt to produce *in vitro* the extreme abnormalities of bone formation shown by the extremities of phokomelic homozygous Creeper embryos. These abnormalities chiefly consist in

(1) a general retardation of cartilage differentiation;
(2) lack of bone formation; and
(3) frequent partial fusion of ulna and radius on the one hand, tibia and fibula on the other, or presence of only one bone in these segments instead of two.

Our problem was to find out if similar abnormalities can be produced *in vitro* by exposing the leg-buds of *normal* embryos to a general retardation of growth. Since it has been found that in phokomelic Creeper embryos the formation of membrane bone is not disturbed, it should be expected that, similarly, any experimental (growth retarding) method by which abnormalities of the long bones comparable to those of phokomelic embryos are produced, would not interfere with membranous ossification *in vitro*. This also was to be tested by experiment.

That our problems might be approached successfully with the tissue-culture method was suggested by earlier observations. It has been shown (Strangeways and Fell, 1926; Fell, 1928) that explants of the undifferentiated mesoderm from the leg-bud of the 3-day chicken embryo formed cartilage during cultivation; but with three exceptions (Fell, 1928) it failed to ossify and was of the small-celled type, similar to that composing the normal avian epiphysis (Fell, 1925); such cultures of small-celled cartilage also failed to synthesize phosphatase (Fell and Robison, 1929). On the other hand, cultures of the 5-day embryonic femur grown under precisely similar conditions not only formed hypertrophic cartilage but ossified and synthesized phosphatase (Fell and Robison, 1929). Since growth *in vitro* with the methods available at present *per se* implies a general retardation of growth, these observations seemed to suggest the existence of a critical period in development before which growth retardation inhibits cartilage differentiation and ossification, while after this point growth retardation does not interfere with these processes of differentiation.

Technique

The cultures were grown by the watch-glass method previously described (Fell and Robison, 1929; Fell and Canti, 1934). In this technique the

explants are placed on the surface of a clot composed of plasma and extract, which is contained in a watch-glass; the watch-glass is enclosed in a petri dish carpeted with a layer of wet cotton-wool, in which a circular hole has been cut to allow of transillumination of the explants for microscopical study. The cultures are incubated at 38·5° C, and the explants are removed from the clot and transferred to fresh culture medium every 48 hours.

The explants were fixed in Zenker's solution containing 3% glacial acetic acid for about 2 hours. Serial sections were cut and the slides were stained with safranin and picro-indigo-carmine, or occasionally with Mallory's triple stain.

The Effect of Growth Restriction on the Ossification of Cartilage

Object of Experiments—To find whether conditions which restrict the growth of the developing limb-skeleton also prevent its ossification.

Material and Methods—The axial condensation of mesoderm destined to form the limb-skeleton was removed from the leg-buds of 91 embryos ranging in age from 3½–5½ days. One rudiment from each pair of buds was cultivated in " normal " medium consisting of four drops of embryo extract mixed with four drops of plasma. The opposite rudiment from the same chick was cultivated in a " growth-restricting " medium which was prepared as follows. One drop of the embryo extract used for the normal medium was added to a tube containing 5 cc of Pannett and Compton's saline with which it was carefully mixed. Five drops of this saline were then mixed in the culture vessel with three drops of plasma, the trace of embryo extract in the saline being sufficient to clot the plasma in a few minutes. In the first of the five experiments made, the growth-restricting medium was rather less rigorous than that described; two drops of embryo extract were added to the tube of saline and the final culture medium was composed of four drops of saline and four drops of plasma. As the results of this experiment were essentially the same as those obtained in the later experiments, they have not been considered separately.

The explants were maintained for a period which, when added to the age of the embryo from which they were taken, equalled the normal incubation period, 21–22 days. Thus the rudiments from 3-day embryos were grown for 18 days, those from 4-day embryos for 17–18 days, and those from 5-day embryos for 16 days. At the end of the culture period each explant was fixed and serially sectioned.

Since embryos of the same age vary considerably in their degree of development a projection drawing was made of one leg-bud from each embryo used, so that the length of the bud before dissection could be ascertained. To provide a record of the growth and behaviour of the explants during cultivation, a projection drawing was also made of each rudiment immediately after explantation and at intervals of 48 hours throughout the culture period.

General Analysis of Results—In order to analyse the results of the experiments, the data were summarized and then tabulated in the following way. The 91 pairs of explants were listed in order of the lengths of the buds from which they were taken, and the presence or absence of ossification in each member of each pair of explants was noted in two parallel columns; thus one of these columns showed the occurrence of ossification in the normal medium and the other its occurrence in the growth-restricting medium.

Some interesting facts emerged from a study of this table. Out of the 39 pairs of explants from buds ranging in length from 0·48–2·19 mm, only five, 13%, of the explants grown in normal medium ossified and only one, 3%, of those cultivated in the growth-restricting medium. On the other hand, out of 37 pairs of explants from 2·24–3·81 mm buds, 27, 73%, of those grown in the normal medium ossified, but only 10, 27%, of those cultivated in the growth-restricting medium. Out of 15 pairs of explants from 3·86–5·24 mm buds, all the explants, 100%, ossified in both the normal and the growth-restricting medium. The results are summarized in Table I.

TABLE I

Class	Length of leg-bud mm	Number of explants pairs	Number of explants ossified: Normal medium	%	Growth-restricting medium	%
1	0·48–2·19	39	5	13	1	3
2	2·24–3·81	37	27	73	10	27
3	3·86–5·24	15	15	100	15	100

Cultivation *in vitro*, even under the most favourable conditions, produces an enormous retardation of growth, and from the above results it is clear that in the earlier stages of development, ossification can be completely suppressed in the great majority of explants, merely by cultivation in the normal medium. At a somewhat later stage, however,

ossification is evidently suppressed with more difficulty, as shown by the fact that most of the rudiments grown in the normal medium ossified, although cultivation in the growth-restricting medium was still sufficient to prevent ossification in most of the sister explants. Finally, a stage in development is reached when the processes of differentiation cannot be arrested even by the less favourable experimental conditions, and accordingly ossification occurred in all the explants in both media.

Classification of Explants—For convenience of description the explants have been grouped into the three classes described in Table I, *i.e.*, 1, explants from 0·48–2·19 mm buds; 2, explants from 2·24–3·81 mm buds; and 3, explants from 3·86–5·24 mm buds.

The range of development of the original leg-buds in each of these three classes is, very briefly, as follows. A 0·5 mm leg-bud is elongated at right angles to the long axis of the future limb, fig. 1*a*, and consists of completely undifferentiated mesoderm with no prechondral condensation. A 2·2 mm bud has become elongated in the direction of the long axis of the limb, fig. 1*b*, and the rudiment of the skeleton is present as a Y-shaped condensation of mesoderm, the tail of the Y representing the femur and the two arms the tibia and fibula. In a 3·8 mm bud the long axis has increased greatly in relation to the diameter and the outline of the skeleton can be distinguished in the living limb, fig. 1*c*. Sections show that the femur, tibia, and fibula are partly chondrified, although at the periphery they are not yet sharply demarcated from the surrounding mesoderm; the metatarsals appear as very diffuse elongated masses of mesoderm. A 5·2 mm bud is similar in shape to a 3·8 mm limb, fig. 1*d*, but the femur, tibia, and fibula are better developed; more matrix has been formed in the cartilage which is covered by a fairly well-defined perichondrium of flattened cells, but the chondroblasts have not begun to hypertrophy and there is no sign of osteogenesis. The metatarsals have just begun to chondrify.

Growth—Owing to the distortion of the long-bone rudiments and the fusion of the joints, it was not possible to obtain very accurate measurements of either the entire explants or of individual long-bone rudiments, so that a detailed study of the growth rate in the normal and growth-restricting media could not be made from the camera lucida drawings of the cultures. The amount of growth which took place in the two media could, however, be estimated roughly from the data available.

Most of the explants cultivated in normal medium elongated to about three times their original length, but some of those from the youngest buds of class 1 increased to as much as six times their original length.

As stated above, cultivation, even in the most favourable medium, produces an enormous retardation of growth. Thus the femur of one of the largest explants from class 3 grown in normal medium, at the end of the culture period had attained a length of only 4·2 mm, whereas the normal femur of a 21-day chick embryo is about 23·3 mm in length. The femora of the explants from class 1 and from the younger buds of class 2

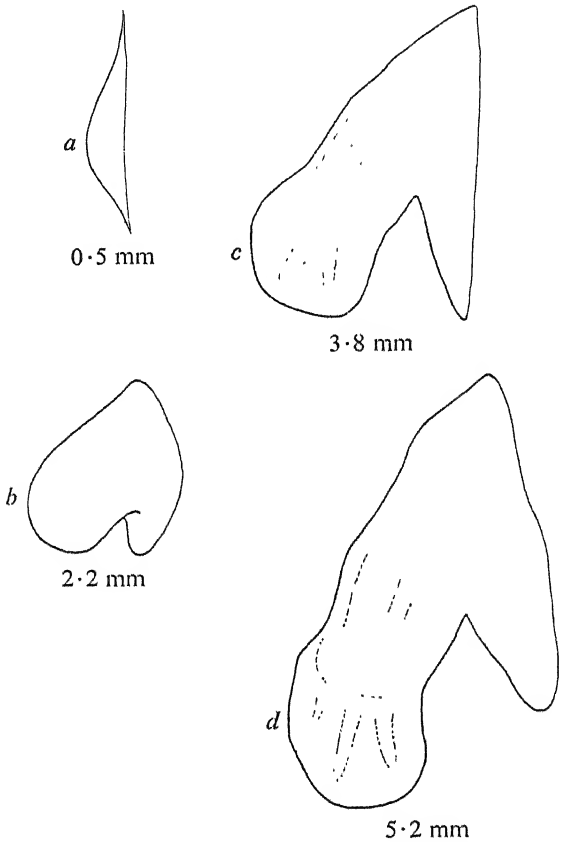


FIG. 1—Projection drawings of normal leg-buds at different stages in the early development of the limb

reached a length which was only about half that of the femora of class 3 explants.

Measurements of the relative lengths of the femora in the explants, grown in the normal and in the growth-restricting medium, showed that the difference in length between the two sets of femora was greater in the explants of classes 1 and 2 than in those of class 3. In classes 1 and 2 the femora of the explants cultivated in the growth-restricting medium

were 20–30% shorter than those of the explants from the normal medium, whilst in class 3 the average difference was only 10–20%. Where one of the femora was ossified and the other was not, the difference in length was much greater than where both femora were either ossified or unossified. The femur was selected for these comparative measurements because it was usually less distorted than the tibia, and fibula.

Anatomical Development—The skeletal rudiments of class 1, 0·48–2·19 mm, when first explanted appeared as oblong blocks of mesoderm, of which the long axis corresponded with that of the original limb. In the older specimens of this group the outline of the femur, tibia, and fibula regions could be faintly distinguished.

After 48 hours' growth the general form of the femur, tibia, and fibula could usually be seen even in the explants from the less developed buds, and by the fourth day the skeleton, which had become chondrified in all the explants, was quite sharply defined. In cultures from the youngest limb-buds the skeleton was usually very imperfect, owing to the damage involved in removing the axial mesoderm from such extremely small, soft buds; in such cases the tarsus and metatarsus were almost always absent, and the femur, tibia, and fibula were usually incomplete. In the explants from the slightly older buds of the group, the femur, tibia, and fibula were often complete and part of the tarsus and of the pelvis were usually present. The pelvis began to appear between the second and fourth day, and chondrified rather later than the distal part of the skeleton. The joints were very sharply marked at the fourth day (Fell and Canti, 1934).

At this stage the rudimentary long-bones were short and relatively thick, but by the sixth day *in vitro* the increase in length had become more marked than the increase in breadth, although the bone rudiments still had a short, stumpy appearance as compared with a normal limb at the same stage of development. This effect, which was particularly pronounced in the explants grown in the growth-restricting medium, was due to the fact that conditions *in vitro* retarded growth much more than they retarded chondrification. As the gross anatomical form of the long-bones is largely the result of differential growth, retardation of the growth rate also involved retardation of anatomical development, so that *in vitro* the limb-skeleton chondrified at an abnormally early stage of anatomical differentiation. Thus the relatively short, thick long-bones in the cultures represented an abnormal persistence of the early embryonic form. It was interesting to note that the longer and thinner a bone-rudiment was at this stage, the more likely it was to ossify later. Associated with this "stumpy" appearance of the shafts of the long-bones was a

smoothness of the articular ends which in the rudiments cultivated in the growth-restricting medium, showed only faint traces of the normal projections and depressions.

After 6 days' cultivation a secondary fusion of the joints began, which was particularly conspicuous in explants from the earlier embryos. In the majority of the explants where the short, stumpy appearance of the long-bone rudiments was very marked, a longitudinal fusion between the tibia and fibula also took place. Fusion both of the joints and shafts of the tibia and fibula usually began earlier and was more complete in the explants cultivated in the growth-restricting medium than in those grown with the strong extract. By the end of the culture period the terminal and lateral fusion of the rudiments was often so advanced that it was almost impossible to distinguish the limits of the original cartilages, and the explant as a whole rather resembled a cricket bat, the femur representing the handle and the fused tibia, fibula, and tarsus representing the blade.

As stated above, the bone rudiments in some specimens were relatively longer and thinner than in others, and such rudiments usually ossified. When ossification occurred in the tibia or fibula, lateral fusion between the cartilages did not take place, although the joints fused as in the non-ossifying explants.

Skeletal rudiments from class 2, 2·24–3·81 mm, often displayed very fine anatomical differentiation when cultivated in the medium containing strong extract. As described above, the original limb-buds contained either precartilage or extremely early cartilage, and the general outline of the skeleton could be seen in the original explants from the older buds. After 48 hours' cultivation in the normal medium, the femur, tibia, and fibula became very distinct, whilst the pelvis, tarsus, and sometimes part of the metatarsus were seen as well-marked prechondral condensations. By the fourth day the entire explant was chondrified, the joints had become very conspicuous and in some explants the pelvis and tarsals were beautifully developed. A comparison of these explants with those of class 1 grown in the growth-restricting medium, showed that on the whole the long-bone rudiments in class 2 were longer, thinner, and more normal in shape, a fact which was correlated with their much higher percentage of ossification. As in class 1, lateral fusion of the tibia and fibula only occurred when both cartilages failed to ossify, although fusion of the joints was invariable.

The explants of class 2, which were cultivated in growth-restricting medium, usually showed a more primitive type of anatomy than the corresponding explants grown with the concentrated extract. As in

class 1, the rudimentary long-bones had relatively short, thick shafts and comparatively smooth and shapeless articular ends, whilst fusion of the joints, and laterally of the tibia and fibula, took place earlier and

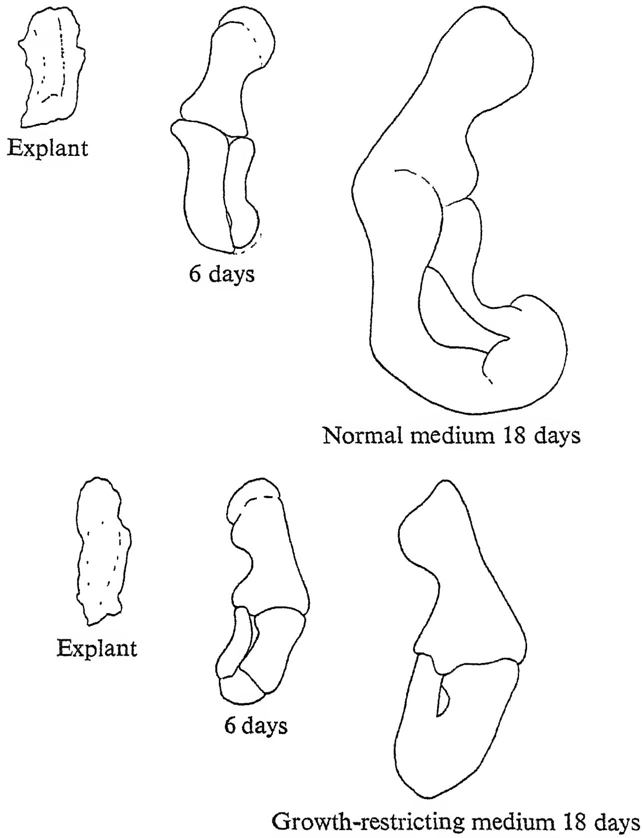


FIG. 2—Projection drawings of a pair of skeletal rudiments from the leg-buds of the same embryo, length of leg-bud: 2·24 mm, at different stages of cultivation *in vitro*. One rudiment was cultivated in normal medium and the other in growth-restricting medium. The tibia and fibula of the explant in the normal medium ossified, but the femur did not; the explant in the growth-restricting medium remained completely unossified. Note the absence of fusion between the tibia and fibula in the ossified specimen and the relatively greater length of these bone rudiments as compared with the tibia and fibula in the unossified specimen. (Sections of these explants are shown in figs. 6–9, Plate 8.)

was more complete than in the corresponding explants in the more favourable medium, fig. 2.

In class 3, 3·86–5·24 mm, the original explants usually consisted of the femur, tibia, fibula, tarsus, most of the metatarsus, and some of the phalanges. The entire limb-skeleton was anatomically fairly well developed

at the time of explantation, but, as compared with the skeleton in later stages of development, the long-bone rudiments were still broad in proportion to their length, and the contour of their articular ends was somewhat indefinite. In the less developed members of class 3 the diameters of the tibia and fibula were almost the same, but in older specimens the tibia was already considerably broader than the fibula.

During the first 4 days in the growth-promoting medium the entire limb-skeleton elongated rapidly. As *in vivo*, the increase in length was relatively much greater than the increase in width. In those specimens in which the tibia and fibula were almost the same width at the time of explantation, the tibia increased in diameter more rapidly than the fibula, so that the size relation between these rudiments altered *in vitro* as it does *in vivo*. The epiphysial regions of the femur, tibia, and fibula developed a comparatively normal shape, and the joints became much more distinct. In the explants from the more developed limbs, the metatarsus acquired a comparatively normal shape, although the phalanges were abnormally short and thick. In the explants from the less developed limbs, on the other hand, the metatarsals were somewhat deformed, the distal ends being thicker than the proximal ends, and the entire metatarsal region appeared large and clumsy in proportion to the rest of the skeleton.

After the fourth day of cultivation the shape of the skeleton became rather less normal, as the bone rudiments often became somewhat bent and distorted especially in the explants from the older limbs.

In the explants cultivated in the growth-inhibiting medium, those obtained from the younger limbs only differed from the corresponding explants grown with the concentrated extract in the relative shortness and thickness of the long-bone rudiments. The explants from the older limbs, however, became much more twisted and distorted than the corresponding explants in the normal medium. This was due to local necrosis in the cartilage, particularly in the tibia and fibula, which thus became softened in places, so that the shaft was readily bent by the growth of the adjacent healthy tissue.

As in classes 1 and 2, the joints began to fuse between the sixth and eighth day of growth and were completely rigid by the end of the culture period. There was no lateral fusion between the shafts of the tibia and fibula, as both these cartilages were ossified in all the explants in both media.

Histology—The non-ossifying cultures from classes 1 and 2 developed essentially the same histological structure both in the normal and in the growth-restricting medium. As described above, the femur was fused

with the tibia and fibula so that in section it was often almost impossible to distinguish the original articular line; usually there was also a partial, and sometimes a complete, lateral fusion between the tibia and fibula whilst the tarsal elements appeared in section as a single mass continuous with the distal ends of the tibia and fibula, figs. 3, 4 and 8, Plate 8, and figs. 15 and 16, Plate 9.

The cartilage cells, which were separated by broad areas of matrix, varied in their degree of differentiation in different parts of the same explant. Some of the unossified specimens contained regions of typical hypertrophic cartilage but, with one exception, these regions were situated in the interior of the cartilage and did not extend to the surface, fig. 5, Plate 8. All stages between this typical hypertrophic, and ordinary small-celled cartilage were encountered in the same explant. The exceptional case in which hypertrophic cartilage extended to the surface, but was not ossified, consisted of a metatarsal which had become completely denuded of soft tissue.

The soft tissue surrounding the unossified cartilage sometimes consisted of a fibrous capsule enclosing the entire explant. In other specimens a fibrous capsule covered the tibio-fibula part, but the femur region was enveloped in a loose connective tissue containing denser fibrous bands and numerous branching cavities lined by endothelium derived from the blood vessels of the original explant. The amount of soft tissue present varied. It was always relatively abundant round the femur, but in many of the explants cultivated in the growth-restricting medium it was scanty or even absent over the tarsal and distal part of the tibio-fibular region.

Small areas of necrosis, usually in the tarsal region, occurred in a few explants, but most of the specimens both from the normal and the growth-restricting medium appeared remarkably healthy.

The ossifying cultures from classes 1 and 2 never showed ossification of all the long-bone rudiments in the same explant. In some specimens the fibula alone was ossified, in others a single metatarsal, in others the tibia and fibula but not the femur, or the femur but not the tibia and fibula, and so on, figs. 6 and 7, Plate 8.

A striking feature of these partly ossified explants was the invariable association of the ossification with hypertrophic cartilage. This was beautifully shown in certain explants where hypertrophy of the cartilage cells had occurred only in a localized area forming a small protuberance on one side of the shaft, which elsewhere consisted of small-celled cartilage; the deposition of bone was strictly confined to the surface of this small hypertrophic region and was completely absent from the much larger

small-celled part, fig. 10, Plate 8. Ossification did not occur, however, unless the region of cellular hypertrophy extended right to the surface of the cartilage and those specimens which, as described above, contained an area of hypertrophic chondroblasts completely enclosed by a zone of small-celled cartilage, fig. 5, Plate 8, were not ossified. The only explant in which cellular hypertrophy extended to the surface of the cartilage, but was not associated with ossification, was that of the metatarsal referred to above, which had become completely denuded of soft tissue so that there was no possibility of the formation of osteoblasts.

In most of the ossified explants of classes 1 and 2, the hypertrophic cartilage extended right across the shaft in the usual way and a zone of flattened cells intervened between the hypertrophic and the terminal, small-celled cartilage as in normal development. The osteoid tissue or bone surrounding the hypertrophic cartilage varied in thickness in different explants. Sometimes it was covered merely by a rather diffuse layer of somewhat flattened osteoblasts, but at others a well-formed two-layered periosteum was present.

In class 3, as stated above, all the explants in both media were ossified, figs. 11 and 12, Plate 9. In most of those cultivated in the normal medium and in many of those cultivated in the growth-restricting medium, the femur, tibia, and fibula and one or more of the metatarsals were all ossified in the same explant, whilst the unossified metatarsals often showed inner areas of hypertrophic cells. In some of the ossified metatarsals, hypertrophy and ossification were localized on one side of the shaft, but in the larger long-bone rudiments, with rare exceptions, the chondroblasts hypertrophied throughout the shaft in the normal manner and the hypertrophic cartilage became surrounded by a layer of osteoid tissue or bone of varying thickness. Both hypertrophy of the cartilage cells and ossification were less advanced in the explants cultivated in the growth-restricting medium than in the corresponding explants grown in normal medium, *cf.* figs. 11 and 12, Plate 9.

Unlike the cultures in classes 1 and 2, those of class 3, especially when cultivated in the growth-restricting medium, usually showed fairly extensive necrotic areas, which was probably due to the relatively large size attained by these explants and the consequent bad nutrition and oxygenation of the inner parts of the tissue.

Conclusion—There is a critical period in the early development of the limb-skeleton during which conditions which restrict growth also prevent subsequent ossification; after this period the same conditions fail to prevent ossification.

The Effect of Growth Restriction on Membranous Ossification

Object of Experiments—To find whether conditions which restrict the growth and prevent the ossification of cartilage also prevent membranous ossification.

Material and Methods—Cultivating the undifferentiated rudiments of membrane bone in a growth-restricting medium involves considerable technical difficulties which for a long time proved insuperable. The 4-day embryonic mandible was selected as the most favourable material, but when it was explanted in a growth-restricting culture medium the tissue failed to spread out on the clot and became completely covered with ectoderm; after about 2 days' cultivation the rudiment became cystic and necrotic. Attempts were made to remove the ectoderm either before explantation or after 2–4 days' cultivation, but, owing to the fact that the future osteogenic tissue occupies a very superficial position at this stage of development (Jacobson and Fell, unpublished experiments), there was reason to believe that the osteogenic rudiment was almost invariably removed along with the adherent ectoderm. Thus, although a few cases of ossification were obtained, most of the explants contained only cartilage and muscle.

A technique was finally devised which in two out of three experiments gave very satisfactory results. The mandible was split longitudinally half-way between the mucous and skin epithelia, the two halves being left attached at the apex of the jaw. The split mandible was then opened out and spread on the clotted medium with the epithelial surfaces of the two halves uppermost and the cut (mesodermal) surfaces in contact with the clot; the excess fluid around the explant was then carefully removed with an extremely fine capillary pipette. In the two successful experiments all the 18 explants spread out into a sheet during the first 48 hours *in vitro* and became firmly attached to the clot by the out-wandering mesoderm. On the second day of cultivation the epithelium could be largely removed by means of a needle and a specially sharpened cataract knife, and was stripped off as a thin membrane with comparatively little mesoderm attached. The explants were transferred to fresh medium at intervals of 48 hours, and more ectoderm was removed in the same way during the first few subcultures. The mandibular rudiment thus treated did not become cystic and necrotic, and it seemed probable that at least some of the osteogenic mesoderm was left intact.

In the unsuccessful experiment referred to above, the plasma used in the preparation of the culture medium happened to liquefy to an unusual

extent and the tissue did not become properly attached to the clot; as a result the epithelium grew completely over the cut surfaces during the first 2 days' cultivation, so that the tissue failed to spread out on the medium and became cystic and degenerate. The experiment was abandoned and only the two successful experiments are described.

In each of the 18 embryos used in the two successful experiments, the skeletal rudiments from both leg-buds were removed in addition to the mandible. One leg-bud from each embryo was drawn with the aid of a camera lucida in order that the length of the bud before dissection might be measured.

The culture medium was the same as that used in the first of the experiments described in the preceding section, and consisted of four drops of plasma mixed with four drops of saline containing a trace of embryo extract. The saline was prepared by adding two drops of embryo extract to a tube containing 5 cc of saline.

The cultures were maintained for 16 days, at the end of which time the explants were fixed and sectioned.

Results—Both the successful experiments gave essentially the same results and are therefore described together. Measurements of the leg-buds from the 18 embryos used, showed that according to the system of classification given on p. 136, 11 pairs, ranging in length from 0·69–1·98 mm, belonged to class 1, and the remaining 7 pairs, ranging in length from 2·24–2·8 mm, belonged to class 2.

Of the 11 mandibular explants from the embryos belonging to class 1, all but one developed membrane bone whilst none of the explants of the rudimentary leg skeleton showed the least trace of ossification, figs. 13, 14, 15, and 16, Plate 9. The seven mandibular explants from the class 2 embryos all developed membrane bone, but only one of the two largest pairs of limb skeletal rudiments showed any signs of osteogenesis and even this was doubtful; all the other limb explants were completely unossified.

When first explanted the mandible consisted of a mass of mesoderm containing neither cartilage, muscle, nor bone. At the stage of development represented by the larger mandibles of class 2, the rudiment of Meckel's cartilage is just distinguishable as a very diffuse elongated mass of mesoderm, but there is no trace of osteogenic condensation; in the mandible at the stage of the class 1 explants there is not even a prechondral condensation. The future myoblasts may be seen as tracts of small, spindle-shaped cells migrating into the jaw around the mandibular nerve Jacobson and Fell (unpublished experiments),

After 2 days' cultivation, the rudiments of Meckel's cartilages, somewhat disorganized as a result of the initial dissection, were distinctly seen as rods and nodules of late precartilage, whilst bands of early myoblasts were also visible. About 24 hours later the precartilage had developed into cartilage and other condensations of mesoderm had appeared in different regions of the explant, which, though somewhat indefinite at first, soon gave rise to compact masses encapsuled by a layer of elongated cells. These later aggregations proved to be ossification centres.

Since watch-glass cultures can only be examined with very low powers of the microscope, it was not possible to follow the progress of ossification in detail, but the appearances suggested that osteogenesis in the cultures was somewhat retarded as compared with the normal process *in vivo*. Osteoid tissue which, to judge by its opacity, was at least partially calcified, was very conspicuous in the ossification centres of 12–14 day cultures. At this stage the nodules of cartilage, which had enlarged considerably, were also very well developed; the myoblasts had formed broad bands of muscle which were often seen to contract, sometimes so strongly as to bend the rods of cartilage. The connective tissue in which the cartilage, bone and muscle were embedded, contained a complex system of spaces lined by endothelium and usually one or more islets of ectoderm.

The explants underwent little further change. Histological sections of the cultures fixed after 16 days *in vitro*, confirmed the observations made on the living tissue and showed the presence of typical small-celled, unossified cartilage, areas of well-developed membrane bone, and beautiful cross-striated muscle fibres, figs. 13, 14, and 17, Plate 9.

The rudiments of the limb-skeleton developed in the same way as the non-ossifying explants described in the preceding section and showed the same type of histological structure, *cf.* figs. 3, 4, 8, and 9, Plate 8, with figs. 15 and 16, Plate 9. The single explant which showed possible signs of ossification was one in which the cartilage cells in the shaft of the femur were very slightly enlarged and were covered by a very thin membrane stained blue with safranin and picro-indigo-carmine, which might have represented a layer of osteogenic fibres. Nothing resembling ossification was found in any of the other explants. A considerable amount of the surrounding mesoderm was purposely left adherent to the skeletal blastema in preparing these explants in order that the entire cartilage might be well covered with soft tissue. Some myogenic tissue was therefore present in most of the limb rudiments and, as in the mandibular explants, formed cross-striated muscle which often showed spontaneous contractions.

Conclusion—Conditions which restrict the growth and prevent the ossification of the limb skeleton do not necessarily prevent membranous ossification.

Discussion

We can now compare the results of the experiments which have just been described with the findings in phokomelic homozygous Creeper embryos. Before doing this, however, it will be well to define the difference between the conditions of our *in vitro* experiments with leg-buds of normal embryos and those under which phokomelia made its appearance. The two factors which, judging from indirect evidence, seem to be of greatest importance in the production of phokomelia, and which were also employed in our *in vitro* experiments, are a retardation of general growth of the embryo and the occurrence of this retardation at a specific period of development. With regard to the retardation of growth it is obvious that the conditions in our *in vitro* experiments were much more drastic than those imposed on the embryo by the Creeper mutation, at least in those experiments in which the homozygous Creeper embryos survived the early lethal stage and lived to develop phokomelia. The ultimate size of the different embryonic bones, even under the most favourable *in vitro* conditions, generally remained considerably smaller than that of the long-bones of phokomelic embryos, *cf.* figs. 3, 4, 6, and 8, with fig. 18, making allowance for differences in magnification. The stage, on the other hand, at which the retarded growth rate begins to influence development of the primordia of the extremities is a somewhat earlier one in the case of the phokomelic Creeper embryos than in the case of the normal leg-buds grown *in vitro*. In the latter instance the leg-buds were allowed to reach a length of at least half a millimetre before they were exposed to the influence of growth retardation, while in the homozygous Creeper embryos growth of the body as a whole has been shown to be retarded at least as early as 36 hours of incubation (Landauer, 1932), *i.e.*, long before the buds of the extremities make their appearance in normal development, and the primordia of the extremities must be under the influence of this retarded growth rate beginning with the time of their first appearance. On the whole, then, it may be said that the earliest stages of normal leg-buds grown *in vitro* came under the influence of growth retardation somewhat later but were retarded to a much greater extent than the leg-buds of phokomelic embryos.

A morphological and histological comparison shows very striking similarities between the effects produced on *early* normal leg-buds, class 1, by growth retardation *in vitro* and the principal features of the

leg-bones of phokomelic homozygous Creeper embryos. We shall confine ourselves to pointing out the most important features of resemblance. Cartilage differentiation is much delayed in the explants of early leg-buds grown in the growth-restricting medium: flattened cells occur rarely or are entirely absent; there is no subepiphyseal zone in which the cartilage cells are arranged in columns (this applies generally to bones grown *in vitro*); cartilage hypertrophy, if occurring at all, is confined to the interior of the shaft, the enlarged cartilage cells never reaching the surface of the bone. All these peculiarities also are characteristic of the long-bones of phokomelic embryos. The same resemblance is found with regard to the lack of periosteal bone formation. Furthermore, one of the most peculiar features of phokomelic embryos, found again in the explants, is the partial or complete fusion of the tibia and fibula. In the phokomelic embryos sometimes only one bone was present in place of radius and ulna and of tibia and fibula, while others showed all stages of fusion. On the whole, the material suggested that a secondary fusion of the anlage material for the two pair of bones had taken place. This is demonstrably true for the explants, and strengthens the belief that the same process takes place in phokomelic embryos. As we compare for each stage the explants grown in the growth-restricting medium with those grown in the ordinary tissue-culture medium we find that those cultivated in conditions less favourable for growth always show the closer resemblance to the phokomelic embryos. On the other hand, we find that there is a definite critical stage before which growth retardation produces much more extreme modifications of differentiation than after it. All this may be taken as an experimental verification of our earlier conclusion that the characteristic traits of phokomelic embryos are produced by a general retardation of embryonic growth acting at a particular period of development. Perhaps the most striking piece of evidence in favour of the conclusion that the peculiarities of phokomelic embryos and of early explants of normal leg-buds grown in growth-restricting medium are due to agencies of the same nature, may be seen in the fact that as the membrane bones were not involved in the phokomelic condition so membranous ossification was not interfered with by culture in growth-restricting medium, even of explants from the earliest embryonic stages, class 1.

It has been shown by a number of investigators that specific abnormalities of different types can be produced in various organisms through the action of environmental (chemical or physical) agencies which retard the developmental rate at a specific stage. The literature on this subject has been reviewed recently by Wright (1934). That similar

effects may result from gene action has been assumed by Wright for the explanation of the origin of otocephaly in guinea pigs. The case of phokomelia in homozygous Creeper embryos (and by implication also of the early lethal effect of the homozygous Creeper condition and of the chondrodystrophic traits of heterozygous Creeper chicks) is, we believe, the first instance in which there is not only definite embryological evidence available for the conclusion that these abnormalities are brought about by an unspecific growth-retarding effect of the mutation in question at a definite stage of development, but in which it was possible also to produce *in vitro* similar structural abnormalities by exposing genetically normal leg-buds to a drastic retardation of growth at an early period of their differentiation. It seems probable that specific genetic traits are produced much more commonly than is realized at present, by general effects on the embryonic organism at a definite period of its development.

Further, the material from the phokomelic embryos and that from the explants discussed in the present report, taken together, seem to afford the basis for some conclusions relative to the mechanism of bone formation in the long-bones of chicken embryos, and these conclusions probably are valid for the ossification of cartilage bones generally. It appears from our material that there is an intimate relationship between the occurrence of cartilage hypertrophy and the formation of bone. The evidence briefly is as follows. Bone is never laid down if only small-celled cartilage is present; this applies equally to the long-bone rudiments of phokomelic embryos and to the explants of long-bones cultivated in normal or growth-restricting medium. In normal development, as well as under *in vitro* conditions, bone formation always occurs *pari passu* with chondroblastic hypertrophy. If, as in the long-bones of phokomelic embryos and in the explants of early leg-buds grown in a growth-restricting medium, hypertrophy of the cartilage cells either does not occur or does not extend to the surface of the bones, no periosteal bone is formed. On the other hand, in these cases very restricted endochondral bone formation may take place adjacent to the islands of hypertrophied cartilage cells in the interior of the diaphysis, fig. 18. That the conditions leading to phokomelia, or the agencies which produce phokomelia-like structures *in vitro*, do not *as such* prevent the formation of bone is evident from the fact that in both cases membranous ossification occurs without disturbance. It appears, therefore, that the suppression of periosteal bone formation *in vivo* (phokomelia) or *in vitro* is a secondary effect of those agencies (retardation of growth rate) which prevent cartilage hypertrophy. Whenever such hypertrophy takes place (*e.g.*, in the interior of the bone shaft), the occurrence of bone formation may be

expected in the neighbourhood of the hypertrophied cartilage. It appears probable that either the differentiation of osteoblasts depends upon the presence of hypertrophic cartilage cells or that certain physiological processes connected with chondroblastic hypertrophy are needed to stimulate osteoblastic activity.

Finally, it should be pointed out that our observations shed some light on the nature of the developmental processes in early chicken embryos. The " critical stage " with regard to ossification and other processes of differentiation and of morphogenesis, which we found in our explants, obviously corresponds to one of the " critical moments " in development to which Stockard (1921) has referred in his work on the experimental production of monsters in fish. The fact that a general growth retardation has the same effect on differentiation in phokomelic Creeper embryos as it has on explants shows that the resulting abnormalities are not due to isolation (*e.g.*, lack of " inducing agencies "), as might be supposed in the case of the early explants, but that they are due to a decrease with age of the susceptibility of parts and organs of the embryo to a general retardation of growth. Evidence of other investigators and observations on the early development of homozygous Creeper embryos, recorded in a previous report, indicate that the extent to which growth retardation interferes with normal development of different parts and organs of the embryo, in part at least, depends upon their developmental rate at the time when growth retardation becomes effective. Our material suggests that rather sudden changes in this susceptibility occur in early development, and this agrees well with the sudden changes of relative growth rate known to occur in different embryo organs after the initial maximum at the time of their formation.

The authors wish to express their thanks to Mr. V. C. Norfield who took the photomicrographs illustrating the paper. They are indebted to the British Medical Research Council and to the Rockefeller Foundation for defraying the expenses of the investigation and to the authorities of Storrs Agricultural Experiment Station for a travelling grant which enabled one of us (H. B. F.) to visit Storrs in order that the experiments recorded in this communication might be planned.

Summary

The skeletal rudiments from both leg-buds were explanted *in vitro* at different stages in early embryonic development; one of each pair of rudiments was grown in a normal culture medium and the other in a special growth-restricting medium.

Very great restriction of growth was caused by both culture media but was greater in the special growth-restricting medium.

Out of 39 pairs of explants from buds ranging in length from 0·48–2·19 mm five rudiments, or 13%, ossified in the normal medium and only 1, or 3%, in the growth-restricting medium.

Out of 37 pairs of explants from buds ranging in length from 2·24–3·81 mm 27 rudiments, or 73%, ossified in the normal medium and only 10, or 27%, in the growth-restricting medium.

Out of 15 pairs of explants from buds ranging in length from 3·86–5·24 mm all the explants ossified in both culture media.

It was concluded that there is a critical period in the early development of the limb-skeleton during which conditions which restrict growth also prevent subsequent ossification, but that after this period the same conditions fail to prevent ossification.

The mandible from embryos with leg-buds ranging in length from 0·69–2·8 mm was then cultivated in a growth-restricting medium along with the skeletal rudiments of both leg-buds.

In 17 out of the 18 mandibular explants, membrane bone developed *in vitro*, but, with one possible exception, none of the rudiments of the limb-skeleton ossified.

From this it was concluded that conditions which restrict the growth and prevent the ossification of the limb-skeleton, do not necessarily prevent membranous ossification.

The early leg-buds of normal embryos, after growing from 16 to 18 days in a growth-restricting medium, show morphological and histological features closely resembling those of the long-bones in phokomelic embryos: there is a general retardation of cartilage differentiation; flattened cells are scarce; the zone of columnar arrangement is not formed; cartilage hypertrophy, if present, is confined to the interior of the shaft; periosteal ossification is absent; tibia and fibula are more or less completely fused. In both cases membranous ossification proceeds normally.

This striking similarity is taken as an experimental verification of our working hypothesis, namely, that the abnormalities of the phokomelic homozygous Creeper embryos are the result of an unspecific growth-retarding effect of the Creeper mutational difference at an early stage of development.

The histological findings in phokomelia and in explants of leg-buds grown in a growth-restricting medium suggest that there is a close relationship between cartilage hypertrophy and the occurrence of bone formation. Ossification does not occur in the absence of chondroblastic hypertrophy.

References

Fell, H. B. (1925). 'J. Morph. Physiol.,' vol. **40**, p. 417.
—— (1928). 'Arch. exp. Zellforsch.,' vol. **7**, p. 390.
Fell, H. B., and Canti, R. G. (1934). 'Proc. Roy. Soc.,' B, vol. **116**, p. 316.
Fell, H. B., and Robison, R. (1929). 'Biochem. J.,' vol. **23**, p. 767.
Landauer, W. (1931). 'Z. mikr.-anat. Forsch.,' vol. **25**, p. 115.
—— (1932). 'J. Genetics,' vol. **25**, p. 367.
—— (1933). 'Z. mikr.-anat. Forsch.,' vol. **32**, p. 359.
—— (1934). 'Bull. Storrs Agric. Exp. Sta.,' No. 193.
Landauer, W., and Dunn, L. C. (1930). 'J. Genetics,' vol. **23**, p. 397.
Stockard, C. R. (1921). 'Amer. J. Anat.,' vol. **28**, p. 115.
Strangeways, T. S. P., and Fell, H. B. (1926). 'Proc. Roy. Soc.,' B, vol. **99**, p. 340.
Wright, S. (1934). 'Genetics,' vol. **19**, p. 471.

Description of Plates 8 and 9

Abbreviations

e.b., endochondral bone.
e.i., ectodermal islet.
e.s., endothelial space.
fe., femur.
fib., fibula.
h.c., hypertrophic cartilage.
m.b., membrane bone.
m.f., muscle fibre.
p.b., periosteal bone.
s.c., small-celled cartilage.
tib., tibia.

Note.—All the sections figured were stained with safranin and picro-indigo-carmine.

Plate 8

Fig. 3—Section of the skeletal rudiment from a class 1, 1·54 mm, leg-bud, after 16 days' cultivation in normal medium. Note the complete absence of ossification and the fusion of the knee-joint and of the tibia and fibula. × 15.

Fig. 4—Section of the skeletal rudiment from the opposite leg-bud of the same embryo, after 16 days' cultivation in growth-restricting medium. This specimen resembles that shown in fig. 3, but is smaller and fusion of the joints and of the tibia and fibula is even more pronounced. × 15.

Fig. 5—Section of the femur of an explant from a class 1, 2·16 mm, leg-bud, after 16 days' cultivation in growth-restricting medium. The femur is unossified but the cells have hypertrophied in the interior of the cartilage; this hypertrophy does not extend to the surface. × 41.

Fig. 6—Section of the skeletal rudiment from a class 2, 2·24 mm, leg-bud, after 16 days' cultivation in normal medium, *see* fig. 2. The tibia and fibula are ossified but the femur is not. Note that there is no lateral fusion between the tibia and fibula although the knee-joint is fused. × 15.

Fig. 7—Same under higher magnification, showing the periosteal bone and well-developed hypertrophic cartilage in the tibia. Owing to the curvature of the tibia the layer of bone is cut tangentially. × 41.

Fig. 8—Section of the skeletal rudiment from the opposite leg-bud of the same embryo, after 16 days' cultivation in growth-restricting medium, *see* fig. 2. The femur,

tibia, and fibula are all unossified and there is lateral fusion between the tibia and fibula. × 15.

FIG. 9—Same under higher magnification, showing the small-celled unossified cartilage of the tibia and fibula. × 41.

FIG. 10—Section of the tibia of an explant from a class 1, 1·58 mm, leg-bud, after 16 days' cultivation in normal medium. On one side of the tibia a restricted area of chondroblastic hypertrophy is seen which is covered by a thin layer of osteoid tissue; ossification stops abruptly at the margin of the hypertrophic cartilage. × 41.

PLATE 9

FIG. 11—Section of the femur of an explant from a class 3, 4·85 mm, leg-bud, after 16 days' cultivation in normal medium. The chondroblasts are hypertrophic and the cartilage is covered by a well-developed layer of bone. × 180.

FIG. 12—Section of the femur of the explanted skeletal rudiment from the opposite leg-bud of the same embryo, after 16 days' cultivation in growth-restricting medium. The chondroblasts are hypertrophic and a layer of periosteal bone has been formed; the bone is less well developed than in the specimen shown in fig. 9. × 180.

FIG. 13—Section of an explanted mandible from a class 1 embryo, length of leg-bud 1·59 mm, after 16 days' cultivation in growth-restricting medium. Two nodules of membrane bone have been formed. × 15.

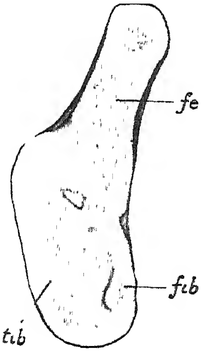
FIG. 14—Same, showing one of the nodules of membrane bone under higher magnification. × 180.

FIG. 15—Section of the skeletal rudiment from one of the leg-buds of the same embryo, after 16 days' cultivation in the same watch-glass as the mandibular explant. This explant is completely unossified. × 15.

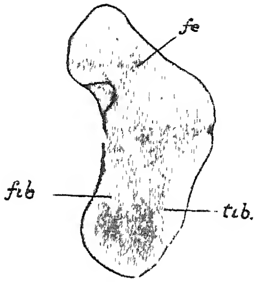
FIG. 16—Section of the skeletal rudiment from the opposite leg-bud of the same embryo, after 16 days' cultivation in the same watch-glass as the two preceding specimens. This explant is also unossified. × 15.

FIG. 17—Section of the explanted mandible from a class 1 embryo, length of leg-bud 1·12 mm, after 16 days' cultivation in growth-restricting medium. Note the cross-striated muscle fibres and the ossification centre. × 300.

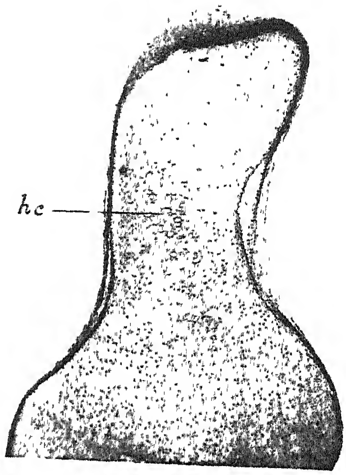
FIG. 18—Section of the femur of a phokomelic chick after 20 days' incubation. There is no periosteal ossification, and chondroblastic hypertrophy is confined to the interior of the femur, *cf.* fig. 5. The hypertrophic cartilage has been partly eroded by ingrowing blood vessels, and connective tissue and slight endochondral ossification is seen. × 30.


fe
fib
tib

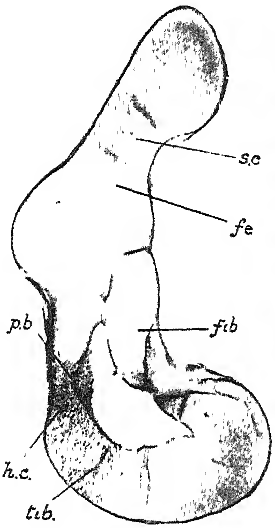
3


fe
fib
tib.

4


hc

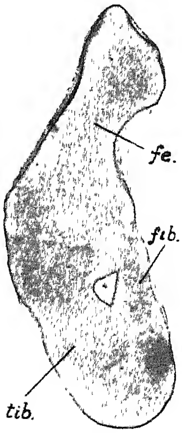
5


s.c
fe
fib
p.b
h.c.
tib.

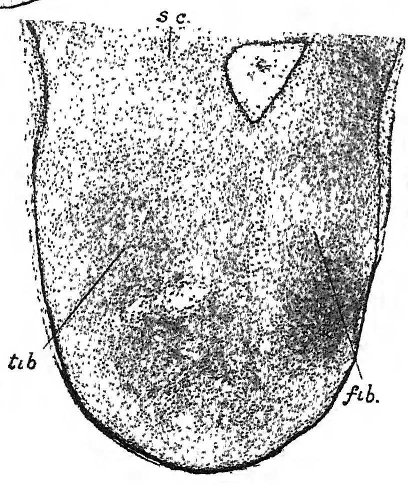
6


hc
pb.

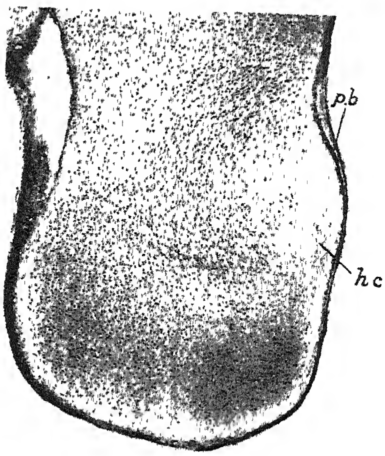
7.


fe.
fib.
tib.

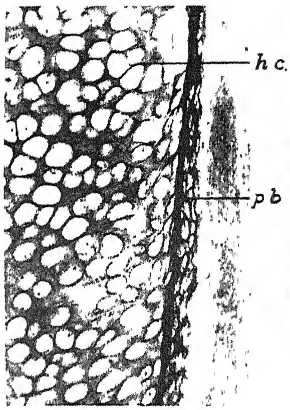
8


s c.
tib
fib.

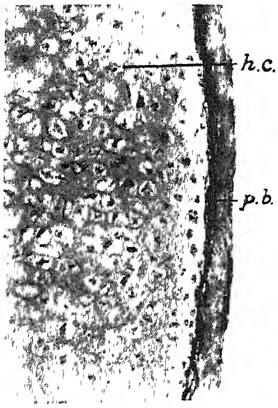
9


p.b
hc

10.

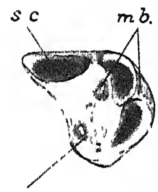


11.



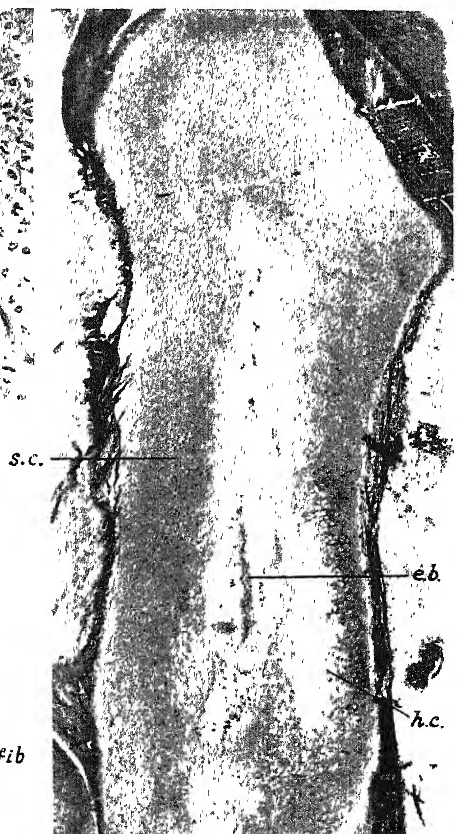
12.

17

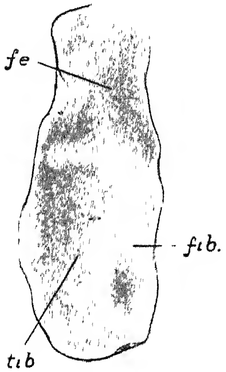


13.

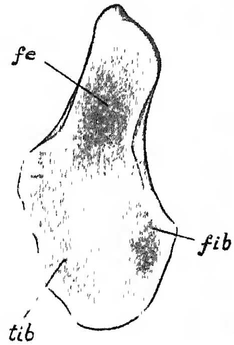
14.



18.



15



16

611.81.611.018.8

The Croonian Lecture

On the Origin and Development of the Nervous System Studied by the Methods of Experimental Embryology

By Ross G. Harrison, Sterling Professor of Biology, Yale University

(*Lecture delivered June* 29, 1933—*Received March* 13, 1935)

[Plates 10–13]

Permit me first to thank the Royal Society for the honour of the invitation to deliver the Croonian Lecture, which I appreciate as one of the highest distinctions that may be conferred upon a biologist. I am reminded, too, of a personal relation that may have something to do with my presence here to-day. Fifty years ago, in 1883, the Croonian Lecture was given by H. Newell Martin, who on that occasion described his epoch-making work on the isolation of the mammalian heart. It was but a few years later that, as an undergraduate at Johns Hopkins University, I attended Martin's lectures on general biology and there received the first inspiration to follow my chosen calling. The awakening of my serious interest in biology was thus due to one who has filled this place before me, one whom I shall always remember with gratitude and respect.

Although many of my predecessors have concerned themselves with the nervous system, no one has considered it in this place from the standpoint of embryology. It therefore seems appropriate to direct your attention to this aspect of the subject to-day, more especially to the part that experiment has played in its advancement. The field covered is almost entirely a development of the twentieth century and is still in the rough. No well-rounded account of it is at present possible, so that I shall not have the satisfaction of showing

"wie wir's dann zuletzt so herrlich weit gebracht."

On the contrary, I approach the task with full realization of its perplexities, but nevertheless with the conviction that the new lines of investigation are leading in the right direction.

Recent advances in experimental embryology, particularly those gained from the study of the amphibian egg, have taken us further and further

away from the preformationist point of view. The cells destined normally to give rise to the central nervous system may be located in the blastula by means of Vogt's vital staining method and may even be projected back upon the unsegmented egg. They require, however, the stimulating action of cells of the substratum after gastrulation, or some other agent, before their potency to form brain and spinal cord can be realized. Furthermore, transplantation experiments show that other cells than those which normally develop into the nervous system may be made to do so by bringing them into contact with this substratum, or " organizer " (Spemann, 1918, 1927). Thus from the earliest stages of development the nervous system is highly regulable, a state which is retained for a long period, possibly as long as the organism is capable of growing and learning.

There is no system of organs in which proper function is so dependent upon the minute arrangement of its cellular elements as is the nervous system, and none that approaches it in the complexity of this arrangement. In no other constituent of the body have studies of structure and of function gone so closely hand in hand. Yet many of the phenomena of development have no obvious relation to function as understood by the physiologist. The relative size of the nervous system in vertebrate embryos reaches its maximum before nervous function begins. Complicated neuro-muscular mechanisms in higher vertebrates are essentially complete structurally before they become active, and in those forms, such as the Amphibia, where activity seems to develop concomitantly with the development of structure, nervous function may be suppressed by chloretone without interfering at all with the development of correlated structures or with their normal functioning as soon as the effect of the anaesthetic passes off (*cf.* p. 187).

The embryo carries on the ordinary functions of organisms, such as respiration and metabolism, but the peculiarly developmental processes are apart from or superimposed upon these and are for the most part continuously changing and irreversible. Nothing is as yet known of the ultimate nature of these changes, but their *loci* may be ascertained and they may be modified in definite manner by known agencies. In other words, they are amenable to experiment.

Scientific theory has advanced through the interpretation of observed facts in terms of physical units. By this means physics, chemistry, and crystallography have reached a high degree of precision and, in a certain sense, perfection. With far less exactness, something of the same kind has been attained in biology, first in the cell theory and later in the theory of the gene, by means of which the phenomena of heredity may be stated

in general terms. Whether we have faith in the reality of these particular units or not, there seems to be something in the human mind that makes it cling to devices of this kind.

In respect to the development of the nervous system, the theory of the gene has at present no direct practical bearing, but the cell theory has, since the nervous system is itself composed of cells. These nervous elements, according to the neurone concept, which is but a special phase of the cell theory, are essentially autonomous in their development, function, and structural arrangement. The embryonic nerve cells, or neuroblasts, appear at first like ordinary cells, but later form long processes or axones (nerve fibres) and dendrites, which connect all parts of the organism according to a definite arrangement.

The cell theory, however, is not without its critics, and the neurone theory, in particular, has been subject to most violent onslaughts. In general, its opponents maintain that cells are not independent but are frequently fused to form syncytia; they stress the view that an organism must be considered in its totality, being more than a mere summation of its parts, and that one of its most characteristic properties is its regulable structure, by virtue of which a part may function as a reduced whole. The latter has been emphasized by one school of experimental embryology and, in the field of neurophysiology, by Bethe in his studies on locomotion in arthropods and vertebrates,* and by Lashley (1926, 1929) in connection with his work on cerebral localization.

This raises a much-discussed philosophical question that need not be entered into here, since the unit theory remains in practice as the most effective instrument of analysis, provided the experimenter recognizes certain complications in the mode of action of the units and regards them as constituents of an integrated whole rather than as altogether independent entities. While, therefore, the neurone theory in its cruder form may be taken as an over-simplification, the fog in which the opposite assumption of a diffuse nerve net is enveloped involves much greater difficulties.

The earlier phases of the development of the nervous system, such as the formation of the neural plate and the closing of the neural folds to form spinal cord and brain, will not be considered here, since they involve general processes of development that are not specifically nervous. The main topics which will be taken up are the following:

(1) The constitution and physical properties of the single nervous unit and its activities in forming the nerve fibre.

* Reviewed in full by Bethe and Fischer (1931).

(2) Responses of nerve fibres to conditions in the medium in which they are growing.

(3) Influences that determine the nerve paths in the embryo, particularly those of peripheral nerves, and the terminal connection between nerve fibre and end organ.

(4) Responses of nerve centres to changed conditions at the periphery and in other parts of the central nervous system.

Neuroblast and Nerve Fibre

The three views regarding the development of the nerve fibre that have been held till recently, and even now have adherents, were all foreshadowed in the middle third of the last century. The oldest, or *cell-chain theory*, according to which the nerve fibre or axone is formed by the same chain of cells that gives rise to the neurilemma sheath, goes back almost a hundred years to Schwann (1839) and has been advocated by a long line of embryologists. The *outgrowth theory*, first enunciated by Bidder and Kupffer in 1857 but much more firmly established by His (1886-1890) and Ramon y Cajal (1890),* constitutes the embryological basis of the neurone concept, according to which the nerve fibre is an outgrowth of a single cell or neuroblast. In the *plasmoderm theory* of Hensen (1864) the nerve fibre is assumed to be a product of differentiation *in situ* of preformed protoplasmic bridges which takes place under the influence of functional activity. As alternatives, the first and second theories concern themselves with the constitution of the nerve fibre, while the contradistinction between the second and third rests rather upon the manner in which nervous connections are supposed to be established. Both pairs of alternatives have been subjected to experimental tests.

The decision between neuroblasts and sheath cell was reached by removing the source of each element in turn and finding that neurones with naked axis cylinders developed when the ganglion crest, the source of the sheath cells, was removed and the neuroblasts were left intact (figs. 1 and 2, Plate 10), while no nerve fibres developed at all when only sheath cells remained (Harrison, 1904, 1906, 1924; Müller and Ingvar, 1921, 1923; Van Campenhout, 1930).

The question of the role of protoplasmic bridges was a more difficult one to dispose of by experiment, for these structures are found everywhere in the interstices between the primitive organs, where the first nerve

* Cajal's papers on the development of the nervous system have been collected in a volume (text in French) published in Madrid, 1929.

fibres run, and their relations to the latter are difficult if not impossible to resolve by purely histological methods. The question, whether the plasmodesmata are actually incorporated into the substance of the axones, or whether they constitute merely a lattice upon which the young nerve fibres are supported in growing out, could be decided only by substituting something else for them.

To test this it was necessary to devise an experiment in which the growing neuroblast was brought to a foreign medium lacking the protoplasmic bridges. A preliminary experiment, in which a small cylindrical blood clot was introduced into the body of the embryo in place of the excised spinal cord, showed that fibres from the rhombencephalon grew out into the clot. The next and final step was to remove the embryonic nervous tissue from the organism entirely by means of the explantation experiment, the method now known as tissue culture (Harrison, 1907).

Pieces of medullary cord from frog embryos, taken before nerve fibres had differentiated, were placed each in a hanging drop of frog's lymph under aseptic precautions and the preparations sealed. After an interval varying from one to three days, fibres of hyaline protoplasm with finely branched amoeboid endings were found extending from the implanted tissue into the clotted lymph, fig. 3, Plate 10. The fibres, at first coarse, were drawn out to fine threads by the amoeboid movement at the ends. The rate of progression and the independence of each fibre from its fellows and from other cells could be readily observed (Harrison, 1910). Detailed comparisons with the earliest nerves in sections of embryos, fig. 4, Plate 10, showed that the hyaline threads were in fact nerve fibres, and appropriate controls made with cultures of other tissues proved that their formation was due to the specific activity of presumptive nervous tissue. These observations have since been greatly extended and are now generally accepted.

Only one conclusion is possible. The substance of the axone originates in the neuroblast and is spun out by means of the amoeboid activity of the end without material contribution from the surrounding protoplasmic bridges, which may serve, however, as a framework to support and guide the fibres. The outgrowth of the nerve fibre is, therefore, a mode of protoplasmic movement and not a mere progressive differentiation *in situ*.

With most other cells of the embryo the neuroblasts share the property of being actively amoeboid. In plasma cultures they send out hyaline processes terminating in fine pseudopodia, which, according to Mossa (1927), are seen to be very long and delicate when observed under dark field illumination. Here the close similarity ends, for the processes of

the nerve cells have a different consistency, with extreme ductility. The usual result of the movement of the amoeboid end is, therefore, not the locomotion of the cell as a whole but the extension of the process into a fine filament. Tension is thus exerted and this may be supplemented by the flowing of the cytoplasm of the perikaryon into the fibre itself or, as Olivo (1927) has occasionally observed, by the actual movement of the whole neuroblast through the culture medium. Both of these types of movement have their counterpart within the embryonic body.

The velocity of outgrowth varies within wide limits. In the amphibian nerve it may attain the rate of 1 μ per minute, though usually not over 15–20 μ per hour. In cultures from the warm-blooded chick embryo Burrows (1910) found that the rate of extension may reach 90 μ per hour. The available data on nerves developing within the embryo or regenerating in adult organisms, which have been tabulated by Williams (1930), indicate velocities of about the same order of magnitude, with variations even wider than those here given. Speidel's observations on the living nerves in the tail fin of tadpoles also fall in line with the above observations. According to Cajal (1928, p. 230), the growth of the regenerating mammalian nerve in the peripheral stump averages from 83 to 125 μ per hour but may attain a rate of 170 μ, which is the maximum recorded.

The first nerve fibres to appear in a culture of embryonic nervous tissue usually grow into the medium from masses of cells, so that individual fibres cannot be traced to their particular cells of origin. However, there are numerous exceptional cases in which whole neurones are found isolated and visible in their entirety. The fibres usually run independently throughout their entire length but may form anastomoses with other fibres, which, however, are often resolved later. In cultures from the chick embryo, where the fibres are of finer texture than in the Amphibia, the formation of nerve nets is more frequent, and they seem often to be of a more permanent nature. Such nerve nets are particularly clear in the sympathetic fibres growing from ganglion cells in the intestinal wall of the chick (Lewis, 1912), but they are also found in cultures of spinal cord and brain, where Levi (1917) observed that they were more frequent when the fibrin clot was not dense, fig. 7, Plate 11. However, the anastomoses, even when they appear complete optically, frequently prove to be but temporary. On the other hand, Bauer (1932) finds that in cultures of the forebrain the formation of nerve nets is the usual occurrence and that the ganglion cells become united with each other by bridges. In the more highly differentiated cultures such structures as end feet (Held) and pericellular nets are formed. Various other observers have contributed to this field, but the observations are conflicting, and it is impossible

at the present juncture to form a correct estimate of their significance.* Undoubtedly many of the discrepancies are due to differences in the source of the material, in its age and in the medium used for cultivation (Mihálik, 1932). According to Esaki (1929), nerve fibres growing from spinal ganglia show numerous anastomoses, while those from the spinal cord or brain have few or none at all.

While the above experiments leave no doubt regarding the primary origin of the nerve fibre as the process of a single cell, we are obviously still confronted with the old and perplexing question, whether nervous connections are made by means of cytoplasmic continuity or by contact. This, in turn, is but part of the more general question of the occurrence of syncytia in the animal organism. Definite proof that the vertebrate nervous system in its primitive state consists of discrete cells was offered more than twenty years ago (Harrison, 1910, p. 793). The relation of the sheath cells to the nerve fibre was also shown to be secondary, and attempts to classify it as syncytial, still frequently encountered, are mistaken. Even the penetration of nerve endings into the cytoplasm of epithelial cells and the fusion of nervous end plates with the sarcoplasm of muscle fibres, so clearly demonstrated by Boeke (1933), are but the final stages in the establishment of intimate nervous connections and in no way disprove the genetic and physiological independence of the nerve elements. Perhaps the most favourable evidence for the existence of syncytia has been given by tissue cultures, but both Lewis (1922) and Levi (1925, *a*), who have examined it critically, practically reject the supposed proofs. Levi points out that even in cases where actual fusion takes place, independent cell territories exist, and that in the supposed nerve nets each fibre is probably dependent upon a single cell.

The existence of neurofibrillae, fig. 7, Plate 11, in nerves grown in culture media was first demonstrated by Burrows (1911) and then by Lewis in the sympathetic plexuses just mentioned. Levi has seen faintly fibrillar structures in living cultures, especially when the nerves are flattened against the coverslip, and it seems possible that they may change their form and arrangement according to conditions in the medium. It is questionable, however, if these are identical with the fibrillae which are revealed by the silver impregnation method.†

The physical properties of living nerve elements in tissue cultures, which have been studied by Levi (1925, *b*) and by Péterfi and Kapel

* For a full discussion of this subject *see* Levi (1934).

† de Rényi (1929, *b*) has demonstrated, by means of micro-dissection, the presence of neurofibrillae in living axones of the lobster, but has been unable to do so in axones of the frog (1929, *a*).

(1928) by means of micromanipulation, are of much interest. According to the former the fibres may be readily stretched without tearing, after which they remain sinuous for a time before returning to their original length. When pressure is applied locally, a swelling arises from which pseudopodia are given off. Similar activities may also occur beyond the point of stimulation and also when the cell-body is touched, fig. 8, Plate 11. If sharply pressed, the cytoplasm of the perikaryon suddenly becomes coarsely granular. Coalescence of two adjacent fibres may be produced by pressing them together and may persist for hours. Péterfi and Kapel (1928) find also that on pricking a young neuroblast with the micro-needle the cytoplasm becomes granular. The cell becomes rounded by withdrawal of its processes, but soon reverse changes set in and, if a new axone is formed, it is always on the side of the cell opposite the point of stimulation.

The observations on the behaviour of fibres that have been cut off from their cells of origin are especially noteworthy. Ingebrigtsen (1913) had previously found that such fibres, like nerves within the body, undergo rapid degeneration, and that regeneration takes place from their central ends. According to Levi, however, the activities of the distal portion are interrupted for a brief period only, after which the movement of pseudopodia at the peripheral end is resumed. The proximal end of the severed process also swells and sends out pseudopodia, so that the fibre may be stretched by pulling from both ends. This activity may continue for as long as twelve hours, but sooner or later it ceases, unless the two cut ends reunite. The fibre is then restored *ad integrum*, almost as if nothing unusual had taken place; fusions of this kind have been observed between fibres that did not originally belong together.

One of the most striking properties of the neuroblast, as seen from the foregoing, is the extreme sensitivity of its growing end to mechanical stimulation, and it is not improbable that this may be connected with the stereotropic responses known to occur in developing nerves (p. 165).

The Responses of Nerve Fibres to Conditions in the Medium

Before taking up the factors that direct the outgrowing nerve fibres within the organism, it will be of advantage to consider the responses made by nerve cells isolated *in vitro*, where the experimentally imposed conditions may be better controlled. Of the various factors assumed to influence the direction of outgrowth, the most discussed are localized

chemical differences in the medium, electrical fields, and the configuration of the solids with which contact is made.

Chemotropism

The theory of chemotropism originated with Ramon y Cajal (1892) in his work on the retina. It was based primarily upon the postulated activity of the growth cones, which he had discovered at the ends of growing nerve fibres and which are identical with the amoeboid endings seen in tissue cultures. Later this was elaborated into a more general theory of neurotropism.* Cajal's conception of chemotropism is pictured vividly in the following sentence: " From the functional point of view the growth cone may be regarded as a sort of club or battering ram, endowed with exquisite chemical sensitivity, with rapid amoeboid movements, and with a certain impulsive force, thanks to which it is able to press forward and overcome obstacles met in its way, forcing cellular interstices until it arrives at its destination " (1899, pp. 544-5).

While it has seemed necessary to make some such assumption—and there are various observations upon developing and regenerating nerves that would be readily explained by it—no crucial proof of chemical sensitivity has ever been forthcoming. Obvious experimental methods of testing it are either the planting of other kinds of cells (muscle or epithelium) in the same culture with nervous tissue or the introduction into the medium of capillary tubes filled with known substances, according to Pfeffer's method, and then noting their influence upon the velocity, intensity and direction of outgrowth of the nerves.

The latter type of experiment, began shortly after the introduction of tissue culture, did not yield definite results (Harrison, 1912), and recent studies by Weiss (1934) have shown that in such experiments unsuspected mechanical strains are more likely to be the primary effective agent than the supposed chemical factors. Tubes filled with extracts of brain, muscle or liver fail to have any directive influence on outgrowing nerve fibres, as do pieces of degenerating peripheral nerves, when proper precautions are taken to avoid vectorial strains on the fibrin as it clots. Centanni and his pupils† have, it is true, described differential growth in such *colture affrontate*, but they were concerned with the growth of connective tissue and sheath cells from explanted pieces of peripheral

* This term was first used by Forssman (1898, p. 96) in his celebrated work on nerve regeneration. For a general account of the theory *see* Cajal (1913, 1928), Tello (1923), and Mangold (1928).

† *Cf.* Bisceglie u. Juhász-Schäffer (1928), p. 160.

nerves and not with nerve fibres themselves. Moreover, no assurance is given that the tubes and tissues introduced into the medium did not exert an influence on the arrangement of the fibrin net.

That the presence of other tissues in a culture may influence the growth and differentiation of nervous elements in other ways has been shown by Grigorjeff (1931, 1932). The vigour and manner of outgrowth of nerve fibres and the type of nerve ending formed are changed by the presence of mesenchyme. Nerve bundles growing over this tissue form fewer anastomoses, and their neurofibrillar structure is more fully differentiated; the nerve endings in contact with mesenchyme cells are more definite. When striated muscle is present, nerve endings within the sarcoplasm, similar to those found in the embryonic body, are formed, fig. 9, Plate 11. Some of Grigorjeff's observations, such as the bending of a nerve fibre to reach a cell, or the convergence of a number of fibres upon a small group of cells, are interpreted as evidence for definite tropisms, possibly of a chemical nature, but here again there is no rigorous proof.

Galvanotropism

The possible effects of the electric current upon outgrowing nerve fibres have been the subject of much speculation and little experimentation.*

Ingvar (1920) reported that when very weak currents were passed through the medium into which small pieces of central nervous system of chick embryos had been explanted, outgrowing cell processes were oriented almost entirely in the direction of the lines of force in the galvanic field. Those growing toward the anode showed morphological differences from those directed toward the kathode. Again, if a weak current was passed through the culture by means of a single conductor, the direction of outgrowth of fibres and cells was perpendicular to the conductor. These observations seemed to confirm the theories of neurobiotaxis and stimulogenous fibrillation.

Experiments by Weiss (1934), using different electrodes and a wide range of current density, have so far failed to confirm these findings, and Weiss

* Already in 1892 Strasser proposed the hypothesis that the motor cells in the embryonic cord spinal send out their processes and move as a whole toward the muscle plate " in the direction of the greatest difference of potential of the electric charge " (p. 734). Hypotheses of this kind have been built up into the theory of neurobiotaxis of Kappers (1908, 1917, 1922, 1933) and the related theory of stimulogenous fibrillation of Bok (1915). It will not be possible to enter here into a discussion of these interesting theories, which, for the most part, are not based upon rigorous experimental proof, and, furthermore, are not in harmony with much of the available experimental evidence.

has suggested that Ingvar's results may have been due to the oriented arrangement of the fibrin, brought about by the wick electrodes used. Moreover, there are serious theoretical difficulties involved in the simple interpretations given in the above observations. However intriguing the theory of galvanotropism may be in relation to the development of the nervous system, it must still be regarded as without substantial foundation.*

Péterfi and Williams (1933) have recently obtained effects of another kind on neuroblasts and axones from the central nervous system of chick embryos through the action of much stronger electric currents.† On stimulation the cell-body of the neuroblast becomes more highly refracting, then granules appear and the cytoplasm withdraws a little from the kathode. Similar granulations appear in axones, and the end bulbs become larger and more diffuse, but when the circuit is broken, there is an after-effect, the axone extending toward the anode. Whether reactions of this kind, in case of more prolonged stimulation, would have any lasting morphogenetic effect remains an open question.

Stereotropism

Inquiry into the possible mechanical factors that may act upon outgrowing nerve fibres has resulted in more definite and satisfactory conclusions. Such factors figured in the earlier accounts of the outgrowth theory and are implied in Cajal's description of the growth cones. They were rendered more probable by the first tissue culture experiments, and crucial evidence was obtained later from the study of the behaviour of cells and nerve fibres in media with different kinds of solid support. Loeb (1902, 1907) had already concluded from earlier observations that stereotropic stimuli play an important part in the movement of epithelial cells. Experiments, in which mechanical and chemical factors in the medium were varied independently, definitely showed that no cellular

* Huzella (1934) has recently reported positive results from growing heart fibroblasts in plasma cultures between the poles of an electro-magnet without material contact with the latter. The intensity of the field was alternately increased and diminished by rotating iron plates placed between the cultures and the magnet. Greater growth takes place in the direction of the magnetic poles, and the individual cells are stretched in the same direction. Whether this is a direct action upon the outgrowing cells or is effected through the fibrillar structure of the medium has not been determined.

† The currents used by Ingvar were exceedingly weak, ranging from 2 to 4 $\times$ 10^{-6} amp. The effects noted by Péterfi and Williams followed the application of currents from 1000 to 14,000 times this strength (2 to 55 $\times$ 10^{-6} amp).

growth or migration occurs except when the cells make contact with a solid body, such as the cover slip, the fibrin net, or fibres (spider web) immersed in fluid, fig. 6, Plate 10, or with the surface film of the medium (Harrison, 1914).* The reactions are characteristic for each form of support, and, in the case of spider web, cells often surround the fibres as sheath cells cover a nerve, fig. 5, Plate 10.

The foregoing tests were made mainly with fibroblasts and epithelial cells, but evidence was obtained that nerve fibres react similarly. Furthermore, the relation of outgrowing nerve fibres in the embryo to solid masses of cells and to the interstitial network bears out the interpretation that they too react to solids and are oriented by the configuration of the latter. It was to be expected, therefore, that further light would be thrown upon this question by the study of the relations of developing nerve fibres to the fibrin net, formed under various mechanical conditions.

Experiments to this end were carried out in the Zoological Laboratory at Yale University by Weiss (1934). Earlier work by the same author (1929) had shown that fibroblasts from the chick embryo respond to the structure of the fibrin clot in which they are cultivated. When a small mass of these cells is explanted to a film of plasma stretched across a frame of suitable size and of the shape of an equilateral triangle or a rectangle, the greater elastic tension lies in the direction of the shortest lines between the mass and the frames. This orients the micellae of the colloidal medium during coagulation and, in these sectors, leads to a parallel arrangement of the fibrillae in the laminated membrane of which the clot is composed, whereas elsewhere, in the absence of a sufficiently strong directive factor, a more diffuse net is formed.† The growth and migration of the fibroblasts is greatest in the sectors which have oriented

* Carrel and Burrows (1911), using cotton fibres and silk in tissue cultures, also called attention to the necessity of solid support for outgrowing cells. Matsumoto (1918) added much further evidence for stereotropism in a study of corneal epithelium in relation to the surfaces over which it grows. More recently Huzella (1931; with Lengyel, 1932) has demonstrated by very ingenious methods the adaptation of growing fibroblasts and other cells to the supporting reticulum. The latter may be either natural (frozen sections of various organs) or artificially prepared by coagulation *in vitro* under special mechanical conditions by the method first introduced by Nageotte (1927, 1931).

† According to Weiss (1929), the fibrillar arrangement in the individual laminae of the film is submicroscopic. However, in Baitsell's (1917) experiments, made under different conditions, a parallel arrangement of fibrillae was just as clearly visible under the microscope when the fibrin clot was subjected to tension as was the diffuse net in regions where tension was not applied.

fibrillae and least in the diffuse sectors, fig. 10, Plate 11. In the former the cells are spindle-shaped and radially oriented, fig. 12, Plate 11, while in the latter they tend to be stellate or multipolar, fig. 13, Plate 11. The more intense growth is due largely to the greater availability of fluid in the sectors with parallel fibrillae, and there is a reciprocal effect, since the local dehydration of the membrane by the rapidly growing cells increases the tension and leads to accentuation of its structural characters.*

In ordinary cultures of nervous tissue the outgrowing fibres are not definitely oriented. If, however, either before or after implanting a piece of nervous tissue from a chick embryo in a drop of plasma, the drop is stroked with a needle or a brush in a given direction, the fibrin threads, as they form on the surface, assume a parallel arrangement. The growing nerve fibres follow this same general direction, though they often appear wavy instead of straight. Such fibres branch very much less than those growing without definite orientation, as already found by various observers in regenerating nerves. There are fewer mechanical obstacles in the fibrin which is clotted in bands.

Two or more pieces of tissue placed not far apart in a single plasma culture lead to the formation of parallel fibrin bundles running between them, fig. 11, Plate 11. In ordinary coverslip preparations this occurs only when the explanted pieces project above the surface. It takes place also in films stretched on glass frames. Under these conditions heart fibroblasts tend to grow and migrate along the fibrin bundles from one piece of tissue to the other, and this has led to the erroneous assumption of chemical or physical attractive forces between the cultures. Similarly, connective tissue and sheath cells grow out from explanted spinal ganglia, and nerve fibres follow in the same definitely oriented path, figs. 12 and 13, Plate 11. When, however, pieces of the central nervous system are taken, there is little or no cell proliferation and the fibres that grow out are not definitely oriented. In such cases the dehydrating action of the proliferating cells and the consequent increase in tension along the lines between the pieces of tissue are lacking. On this account the structure of the clot is not sufficiently differentiated vectorially to influence the direction of outgrowth of the axones.

Plexus formation may be brought about by implanting embryonic nervous tissue in a plasma medium and surrounding the first clot by a similar medium of different density. When the outgrowing nerve fibres

* According to Huzella (1929), the fibroblasts, as they move along over the fibrin, form a gelatinous secretion which condenses into the argyrophile reticulum, superimposed upon the fibrin net and ultimately replacing it.

reach the border zone between the two media, they are in part deflected in the direction of the boundary, fig. 14, Plate 11, and are thus brought into contact with other fibres, only to bend again and run through the outer zone of the medium. Similar effects are produced by the partial liquefaction of the clot, which often occurs in the zone immediately surrounding the grafted tissue, fig. 15, Plate 11.

The foregoing descriptions show some of the ways in which the configuration of the medium may affect the direction of growth and the arrangement of cells and nerve fibres. They show at the same time some of the possible complications of relatively simple conditions and the great difficulty of separating chemical, electrical, and mechanical factors even in such a simple environment. The evidence for the direct action of chemical and electrical factors is meagre and not rigorously proved. While the evidence for the influence of the structural arrangement of the medium, both visible and ultramicroscopic, rests on a firmer foundation, it is not certain whether such factors act directly or through the mediation of induced conditions of some other nature.*

Nerve Paths and Connections within the Organism

Observations on Living Material

The study of structural relations in the embryo at the time when the first nerve fibres are growing out suggests that factors similar to those outlined above play a role in determining their course. Special attention should be called to some of these, such as the tendency of the fibres to grow in a straight line unless deflected by some obstacle, their predilection for surfaces of solid structures and grooves between them, their guidance by the finer structure of the interstitial tissue. It is important, however, to verify inferences from observations upon preserved material by the study of actual living fibres within the organism. Up to the present this has been accomplished in but one object, the tail fin of the amphibian larva.

The nerves in the fin fold have engaged the attention of many investigators since the time of Schwann (1839) and Kölliker (1846). In living material the independent movements of sheath cells along the fibres were observed some years ago by Harrison (1904, 1924). Now the notable observations by Speidel (1932, 1933) have given us detailed information regarding the outgrowing sensory nerve fibres themselves and the behaviour of the sheath cells, including their relation to the process of myelinization.

* For an able discussion of these questions *see* Weiss (1933).

Growth cones at the ends of nerve fibres behave in much the same way as in tissue cultures, and there can be no question but that they are the active agent in drawing out the axone. Their advance through the mesenchyme of the fin fold is a halting one. At times it may reach 1 μ per minute, but it is usually much slower and sometimes almost ceases. Temporary obstructions may call forth the production of varicosities, giant growth cones or actual branching, and a prolonged encounter with an insuperable obstacle may be followed by retraction of the growth cone or even by its autotomy. After the pioneer fibres are laid down they are followed by others which glide along them, thus indicating a stereotropic response. Anastomoses are frequently formed between neighbouring nerves, and a fibre may double back on a nerve bundle and run centripetally, so that recurrent fibres may meet and pass others growing in the opposite direction, fig. 16. This last observation is of special importance in excluding electrical or chemical gradients as possible factors in directing the movement.

In the formation of the myelin sheath, axone and sheath cells react together, and the latter behave in a characteristic way. The myelin is laid down section by section, and in a given segment it first appears on a level with the nucleus of the sheath cell. The process extends along one fibre of a bundle, while the others may remain myelin free, fig. 17, but frequently the myelinization is discontinuous, in which case a myelin segment is added later to fill the gap. Sheath cells frequently move from non-medullated fibres to myelin-emergent fibres but very rarely in the opposite direction.

The nerve fibres of the tail may be cut either singly or in small bundles and their subsequent behaviour observed (Harrison, 1908; Williams, 1930; Speidel, 1933). Degenerative changes, which occur rapidly in the peripheral stump, involve neurilemma, myelin sheath and axone. Regeneration takes place from the central stump through the activity of new growth cones, the regenerating fibres frequently following the degenerating peripheral tract but rather more often breaking new paths. The velocity of this secondary outgrowth, like that of the primary, varies within wide limits, but the mean is considerably higher when the new nerve is growing in the old sheath (Williams).*

The question of healing of the individual fibres *per primam* (Harrison 1908) has been answered negatively by Williams, who finds that the

* In the spinal nerves of *Hyla* the mean velocity is $7 \cdot 1 \pm 0 \cdot 77$ μ per hour in the old sheath and $2 \cdot 9 \pm 0 \cdot 25$ μ outside, while in the lateral line nerve the corresponding figures are $9 \cdot 9 \pm 1 \cdot 36$ μ and $3 \cdot 1 \pm 0 \cdot 22$ μ respectively.

rapidity of outgrowth is sufficient to explain the phenomena observed. Speidel's observation of recurrent fibres in the fin nerves might also account for the appearance of primary healing when such a nerve is

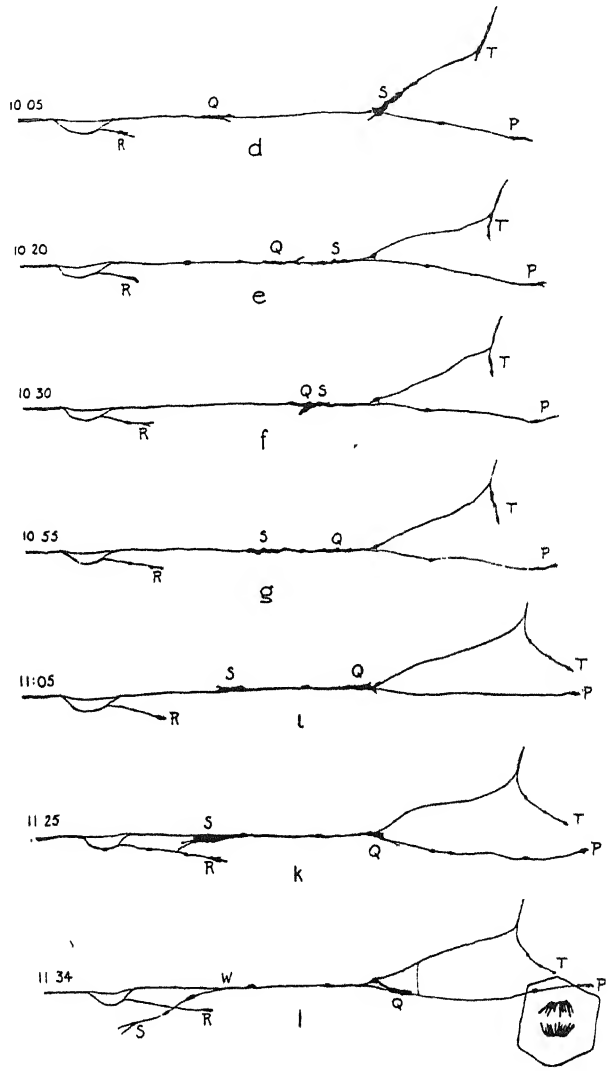


FIG. 16—Growth of two nerve fibres (Q and S) in opposite directions along a third fibre. The fibres meet in (*f*) and pass. Time intervals given on the left. Observations on the regenerating tail fin of a larva of *Hyla crucifer*. After Speidel (1933).

cut, for in these cases both ends are in part central stumps. In spite of these explanations, the possibility of primary healing must be admitted in view of Levi's (1925, *b*) observations on isolated fibres in tissue cultures.

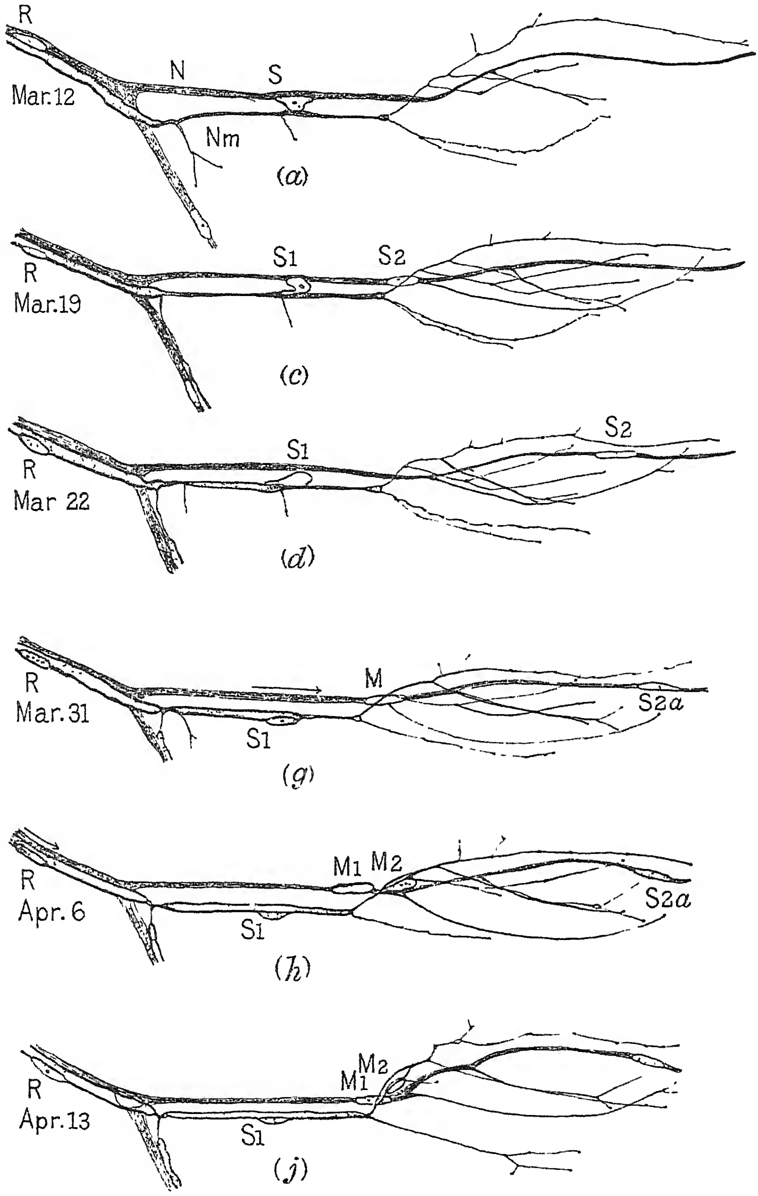


FIG. 17—Myelinization of a sensory nerve fibre in the tail fin of a larva of *Hyla crucifer* observed in the living animal. Two nerve fibres, one the continuation of a myelinated fibre (myelin emergent, *Nm*) and the other *N* of a non-myelinated fibre. The sheath cell (*S*) touches both in (*a*), then divides, one (*S*2) remaining on *N* and the other (*S*1) gradually withdrawing to *Nm* (*c* and *d*). The latter then adds another myelin segment in relation to *S*1 (*d–h*). A second cell (*M*) moves out upon the fibre *N* and divides into *M*1 and *M*2; the latter moves over to *Nm* and another myelin segment is laid down. Observations on a normal living tadpole. After Speidel (1933).

Polarity of Neuroblasts and Conditions first encountered by Outgrowing Fibres

By virtue of the protoplasmic movement which takes place at their ends, the nerve fibres extend to all parts of the embryo. They must move in paths which conform to the topography of the primitive organs, for the distribution of nerves in any species of animal is remarkably constant, the variations being of a minor nature.

At the time when the first peripheral fibres (pathfinders) are laid down, the absolute distances they have to grow are very short and are of the same order of magnitude as the length of isolated neurones actually observed in tissue cultures. Their initial course is usually straight, and the configuration of the surrounding organs is, on the whole, sufficient to account for the deflections observed. The sensory spinal nerves from the dorsal or Rohon-Beard cells run at first laterally, some branches enter the myotemes as proprioceptors (Coghill, 1929) and the others, on reaching the skin, are deflected dorsally or ventrally and to some extent headward or tailward to form anastomoses with branches from neighbouring segments. Motor nerves reach the myotomes almost immediately on leaving the spinal cord, some entering and others bending dorsally or ventrally. Motor anastomoses are apparently not found except in the limb plexuses, and here the peripheral distribution in relation to segmental origin varies considerably. Sensory fibres from the spinal ganglia, which arise later than those from the dorsal cells, run at first with the motor nerves, and fibres of the dorsal rami extend into the fin fold. The olfactory nerves have but a short distance to cover between the nasal placode and the brain, and the optic fibres use the optic stalk as a pathway. The ganglia of the lateral line and the ear develop in close association with the sensory epithelia; in the case of the former the first axones develop before the main placode starts on its long journey from head to tail. This is the most remarkable case of passive stretching known in the development of nerves.

Within the central nervous system the conditions are different in so far as the outgrowing fibres have to pass through a more solid matrix. In the salmon embryo, where the early relations of the neuroblasts are shown clearly, there is evidence that the nerve processes bore their way through the supporting cells,* fig. 18, *gc*. At first rounded and lying in the

* This was pointed out by Harrison (1901) and is confirmatory of the view expressed previously by von Lenhossék. It has also been accepted by Coghill (1929, p. 54) on the basis of observations on *Amblystoma*. Weiss's observations on nerves in tissue cultures (1934, p. 433) are interesting in this connection.

marginal veil, fig. 19 A, *e*, the neuroblasts transform themselves either into bipolar cells, figs. 19 A, *f* and 19 B, *g* and *h*, with processes running up and down the cord, or into unipolar cells with a single process running at right angles to the longitudinal fibres, fig. 18, *com.* The latter are

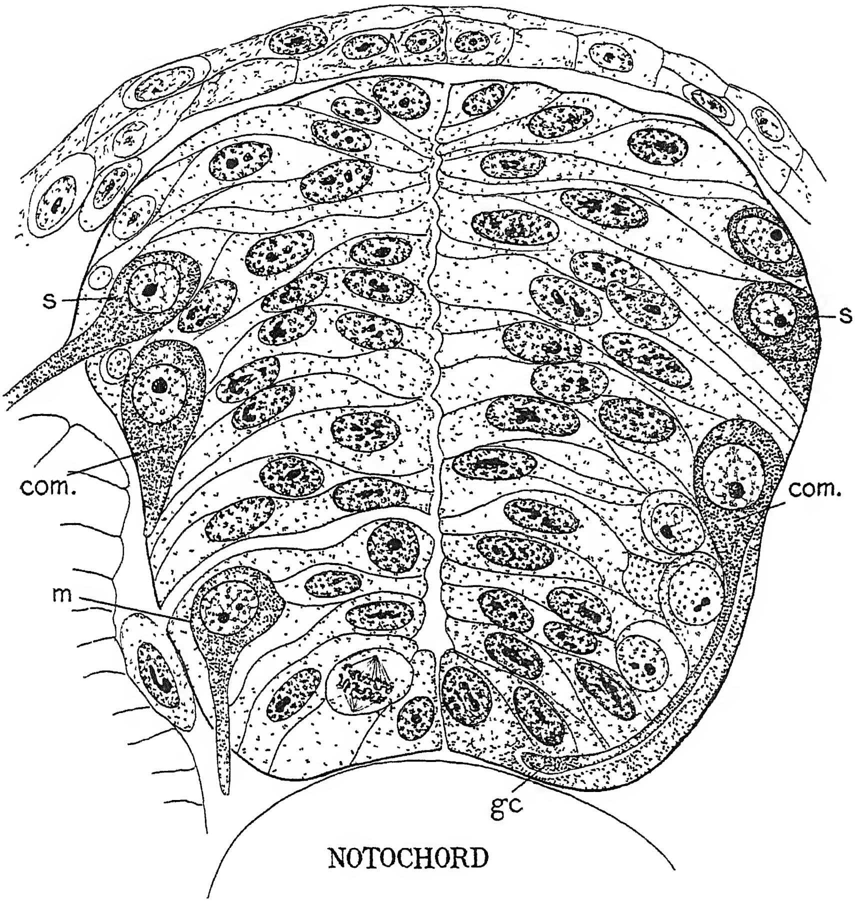


FIG. 18—Composite cross-section of the spinal cord of a salmon embryo with about 29–33 somites. On the right are two Rohon-Beard sensory cells (*s*) and a large commissural cell (*com*) with process running nearly to the mid line, ending in a growth cone (*gc*). On the left are a sensory cell (*s*) with peripheral fibre, a commissural cell (*com*), the axone of which is cut off, and a motor cell (*m*) with axone emerging from the cord. Modified from Harrison (1901). × 932.

either commissural cells, connecting the two sides of the cord, or motor neurones, fig. 18, *m*, which break through the external limiting membrane and reach the muscles. The primary peripheral sensory fibres arise directly from cells of the dorsal column, fig. 18, *s*, or by branching from

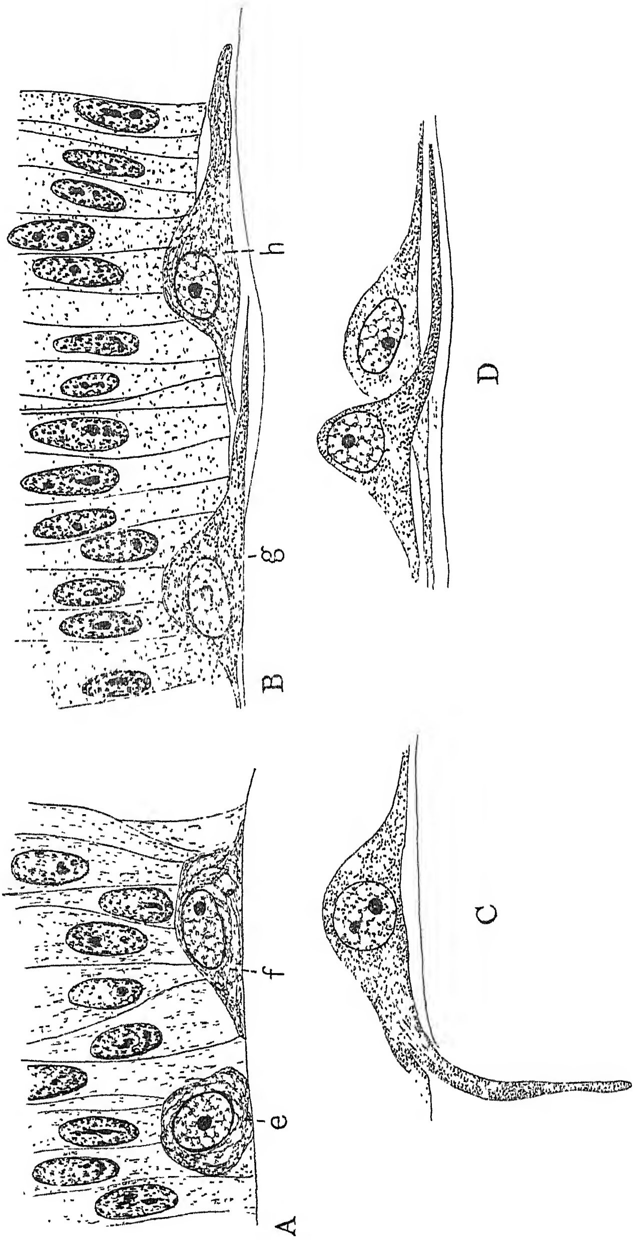


FIG. 19—Cells shown in oblique longitudinal sections of the spinal cord of the salmon embryo. A, from an embryo with 24 somites; two neuroblasts (sensory) embedded in the ependyma, one (*e*) polygonal and the other (*f*) showing beginning of longitudinal processes. B, from an embryo with 31 somites; two similar cells, with well-differentiated processes. C, single dorsal sensory cell (Rohon-Beard) with ascending and descending processes and peripheral fibre running off from the latter; from an embryo with 34 somites. D, two sensory cells from the dorsal column of an embryo with 33 somites. After Harrison (1901). $\times 932$.

the fibres of the dorsal longitudinal bundles, fig. 19 C. Coghill (1913, 1914) has shown that in the urodele, *Amblystoma*, both motor and sensory peripheral fibres arise as collaterals from funicular fibres, fig. 20. Dendrites and other collaterals develop later in time to effect the better co-ordinated movements of the swimming stage.* According to Coghill, differentiation of neuroblasts begins in localized centres and other fibres tend to grow towards these active foci.

The outgrowth of a given neuroblast is not necessarily dependent upon the specific qualities of its surroundings, as is shown by tissue cultures

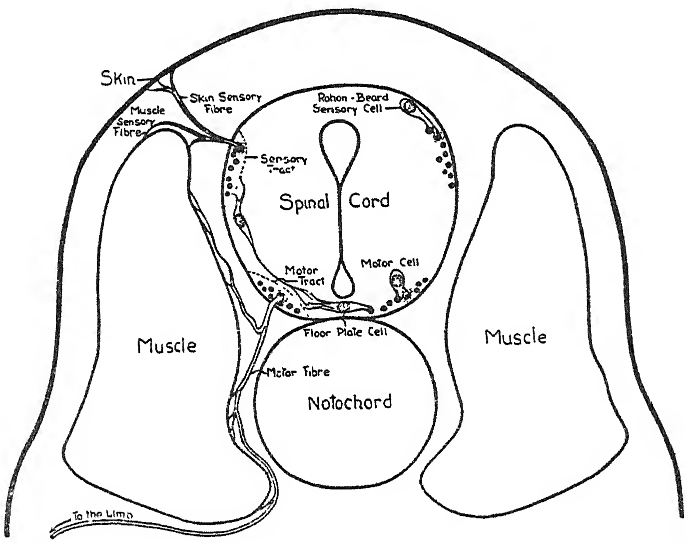


FIG. 20—Diagrammatic cross-section of the spinal cord of an *Amblystoma* embryo showing the relation of peripheral fibres to skin and muscle and certain of the primitive connecting neurones within the cord. After Coghill (1929).

and by experiments in which the surroundings of growing nerves are radically altered. In the former the nerve fibres grow radially from the explanted tissue into the medium; in the embryo they tend to preserve their original direction of growth. For example, when the spinal cord is removed in a frog embryo with neural folds just closed, longitudinal fibres, proceeding from the medulla, run in the same direction through the mesenchyme, a tissue of very different texture, that fills the space normally occupied by the excised cord (Harrison, 1904, 1910). The olfactory nerves likewise grow into the mesenchyme after removal of the

* This most important work of Coghill on the correlation of structure and function in the developing nervous system has been brought together in his University College Lectures, published in book form, 1929.

brain or after transplantation of the nasal pit (Lewis, 1907), and many cases have been observed in which nerve fibres grow for long distances in strange surroundings from pieces of embryonic brain or spinal cord transplanted to other regions of the body (Harrison, 1906, 1910, *a*; Lewis, 1907). Hoadley (1925) has made similar observations on nerves from grafts of the midbrain of the chick growing on the chorio-allantoic membrane.

The foregoing observations indicate that the primary direction of outgrowth of the axone is already determined in the neuroblast, but what determines it is unknown. Most of the explanations that have been given must be relegated to the limbo of pure hypothesis. The earliest visible polarization of the cells in the medullary tube lies in the direction, external limiting membrane, central canal, which corresponds to the surface-depth axis of the medullary plate. Visible differences between the external and internal zones of the cells may be detected in the prenervous stage when the plate is forming, but there is nothing to prove that these apparent differences are due to anything more than the respective relations of the two surfaces, the one exposed to the external world and the other adjacent to the deeper layers of the embryo.* When the axones begin to grow out from the neuroblasts, they are found in almost all cases to extend from the side of the cell adjacent to the external limiting membrane (Mall, 1893),† but some (primary sensory neurones) grow longitudinally along the membrane, while others (peripheral motor nerves) grow directly toward the membrane and perforate it, and again others (commissural cells) are deflected by the membrane to run dorsoventrally or transversely. The result of this is that, in spite of the uniform polarity of the cells, the axones seem to run in all directions, figs. 18 and 19.

* Mall in his study of the histogenesis of the retina (p. 417, footnote) states: " The general indication is that the pole (of the neuroblast) which gives rise to the nerve fibre is the original basal end of the cell." He suggests that cells for which this rule does not hold, for example the cortex cells in the mammalia, have undergone a rotation from their primitive position. Cajal (1908, p. 100) has described in the medulla of the chick certain *neuroblastes intervertis*, in which the axone first grows toward the ventricle only to bend later 180° to run back through the brain substance toward its outer layer.

† Recent experiments by Luther (1934), in which ectoderm of amphibian embryos was transplanted with its inner surface toward the outside, indicate that the surface-interior polarity is not fixed until the neurula stages, and cast serious doubt upon the assumption of a fixed polarization, in this direction, of the elements of the neural plate. In *Triton* the indifferent condition lasts until the neural folds close; in the axolotl, until shortly after the formation of the plate.

There is also a pre-nervous differentiation of the neural plate with reference to the antero-posterior and the medio-lateral axes, and this is controlled by the material underlying it, as shown by Spemann's (1918) rotation experiment with the upper hemisphere of the early gastrula. When the first longitudinal fibres develop in the medullary cord, the direction of their outgrowth takes place in accordance with the antero-posterior polarity; sensory neuroblasts in the dorsal part send out their axones toward the head and motor neuroblasts in the ventral part send theirs in the opposite direction.* In the case of the former, however, it is not possible at first to distinguish, histologically, between dendrite and axone.

The polarity of the medullary cord, when first established, is labile, and even after the neuroblasts have undergone a certain degree of differentiation, their functional polarity may be changed, as Hooker (1917) has shown by reversing segments of the spinal cord in the frog embryo. In such experiments the neuroblasts within the graft develop at first normally in accordance with their original orientation, and there is evidence of some resistance to fibres growing into the reversed segment or from it into the normal cord, where two like poles are apposed. Muscular movements, controlled by the reversed piece, are at first poorly co-ordinated with those of head and tail, but function improves later and may become normal, indicating that the primary morphological polarity of the neurones has been overcome functionally, and that the fibres in the longitudinal bundles which originate in the reversed graft conduct in a direction opposite to normal. After similar operations on younger *Amblystoma* embryos, the reactions are normal from the beginning (Detwiler, 1923).

When a short segment of the spinal cord of an *Amblystoma* embryo is excised and replaced transverse to the body axis, the graft maintains its original polarity, and the anterior part of the nervous system re-establishes continuity through the growth of descending fibres which enter the transverse graft near its original anterior end and pass through it to the posterior part of the cord (Wieman, 1922, 1925). Only then do the ascending sensory tracts from the posterior part of the cord grow into the graft. The fibre tracts change their direction of growth both on entering the graft and on leaving it.

After rotation of a segment of the spinal cord 90° or 135° about its

* Coghill (1929, pp. 55-58), in agreement with Child (1921) and Herrick (1924), refers the polarity of the neuroblasts to the metabolic gradient of the embryo. It seems to me more likely that the two are but separate manifestations of a more fundamental polarity of the protoplasmic units.

longitudinal axis, the outgrowing fibre tracts adjust themselves to the changed conditions by undergoing torsion sufficient to bring them into line with the proper sectors of the rotated piece. After rotation of 180° the adjustment is not so perfect, though evidence of dorso-ventral decussation of fasciculi has been found (Hooker, 1930).

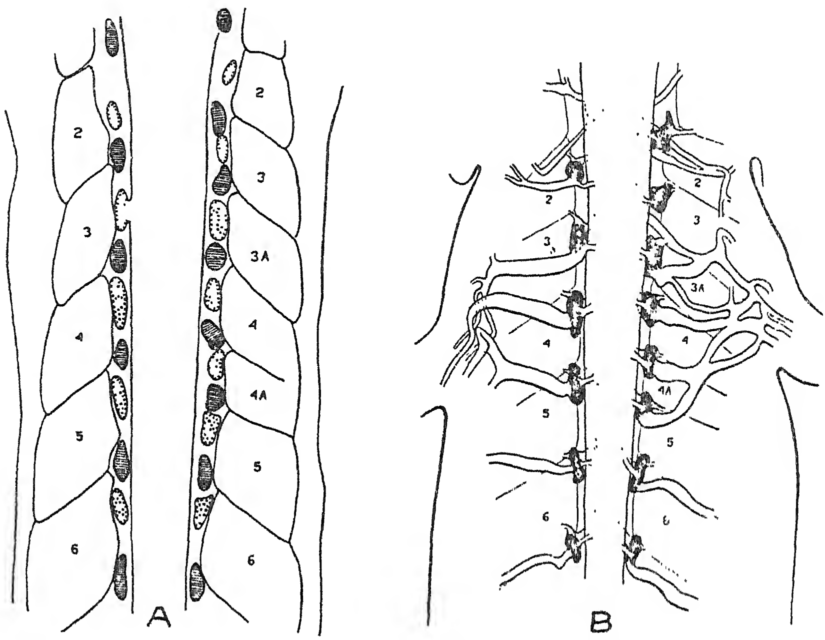


FIG. 21—A, frontal section through an *Amblystoma* larva with two additional somites (3*A* and 4*A*) on the right side which have developed from grafted unsegmented mesoderm; corresponding to these are two additional vertebral arches (striped) and spinal ganglia (stippled). B, reconstruction of spinal nerves in the same case. Five segmental nerves supply the fore limb on the side of operation and only three on the normal side. After Detwiler (1934). × 16.

Peripheral Factors, Especially Those Concerned with the Innervation of Limbs

The segmental arrangement of the spinal nerves is dependent upon the muscle plates, as first shown by Lehmann (1927). However, when the myotomes are completely excised in a region, the spinal ganglia, according to Detwiler (1932, 1934), do not fail altogether to develop but are irregular, and the nerve roots also, though to a less degree. When shorter segments from further tailward are transplanted to the region of the anterior limb, more than the normal number may be inserted into a given space. The number of nerves then tends to correspond to the number of muscle

segments, fig. 21 A, but not always exactly, since the ganglia and the vertebral arches are sometimes less in number than the myotomes. In the above cases the limbs are innervated by all of the nerves of the region, fig. 21 B, and function properly, no matter if the nerves are irregular or are more numerous than in normal cases.

Experiments in transplanting embryonic limbs, begun by Braus (1904, 1905), have thrown much light on the problems of nerve plexuses and peripheral distribution, figs. 27 and 28, Plate 12. Two fundamental facts were discovered in the earlier experiments in this field: transplanted limbs tend to receive their innervation from the nerves belonging to the region of implantation; the intrinsic arrangement of the nerves within the limb is normal. Interpreted in the light of the outgrowth theory, these facts offer an explanation of the segmental variation in nerve plexuses, which, as Fürbringer long ago pointed out, may be coupled with constancy of peripheral distribution. They show that there are two independently varying factors affecting the innervation of the limb. The first, which determines the source of nerve supply, is the position and extent of the limb bud at the time the nerves enter. The second is the mode of segregation and growth of the individual structures of the limb; this fixes the mode of intrinsic distribution (Harrison, 1907, p. 277).

That the problem of the plexus is not so simple as this was shown later by the more extensive studies of Detwiler (1920, 1922), in which limb buds were implanted systematically from one to five segments caudal to their normal position (in extreme cases opposite the eighth, ninth, and tenth somites), or from one to two segments anterior to this in the region of the gills. If the position of the limb were the only factor involved, the nerves entering should correspond exactly to the region of implantation, but this is by no means so. Limbs moved one or two segments tailward receive the normal brachial nerves, and it is only when the graft is placed from three to five segments back that there is a shift in the nerves entering it. There is also a tendency to a greater spread in the nerve supply in these cases, four nerves frequently entering instead of three. If the limb bud is displaced anteriorly into the gill region, the plexus may become slightly shifted in that direction—there are only two spinal nerves anterior to the brachial plexus—but here again the shift in the plexus is less than the displacement of the graft. When the normal limb bud is left intact or allowed to regenerate, and the graft is placed so near that the brachial nerves may reach either bud, the nerves tend to divide and go to both.

The foregoing points to the conclusion that the rapidly proliferating limb bud gives rise to conditions that direct the growing nerve fibres, and that the nerves normal to the limb are thereby affected more than others.

That the attraction to the limb bud is not specific is indicated by further experiments of Detwiler (1928) and Detwiler and Vandyke (1934) with optic vesicles or nasal placodes, instead of limb buds, grafted to the side of the body, fig. 26, Plate 12. In such cases the spinal nerves also tend to run toward the graft, fig. 29, with which, however, they usually form no nervous connection.*

In explanation of these observations, Weiss (1934, p. 430) has suggested that the rapidly growing limb bud or sense organ may alter the density and arrangement of surrounding interstitial structures with which the nerves come into contact when growing out, and that this may facilitate their growth in the direction of the graft (p. 167).

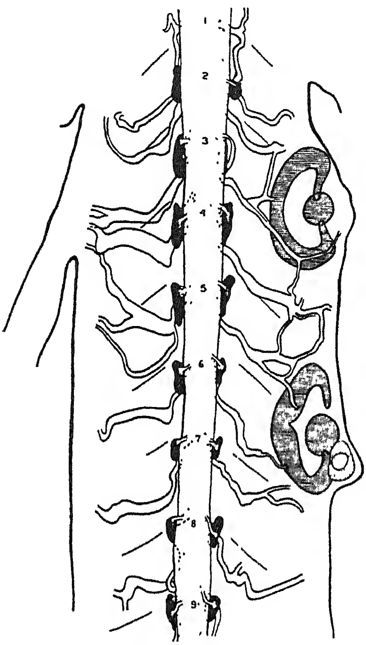


FIG. 29 — Reconstruction showing relation of spinal nerves to two grafted eyes, fig. 26. Branches of the fifth and sixth nerves penetrate the sclerotic cartilage. After Detwiler and Vandyke (1934). × 16.

The grafted limb does not function normally except when fibres from at least one of the segmental nerves of the brachial region contribute to its innervation (Detwiler, 1925; Detwiler and Carpenter, 1929), even though it be well provided with other nerves. Co-ordinate movements require a direct connection with the normal centres in the spinal cord, which, however, need not be nearly so extensive as it normally is. When two or more limbs receive nerves from the brachial plexus, they exhibit synchronized movements, homologous muscles contracting simultaneously in each member. The explanation of this is too controversial at present to be entered upon here.

That the intrinsic distribution of nerves within the limb is due to factors inherent in the limb itself, follows from the fact that a normal arrangement of nerves is found in limbs which have developed under a great variety of circumstances. Grafted to the head, fig. 27, Plate 12; a limb receives its supply from one or more cranial nerves (Braus, 1905; Detwiler, 1930; Nicholas, 1929, 1933); a

* Wieman and Nussmann (1929) and Nussmann (1931) made similar experiments but concerned themselves with the question of the actual innervation of the graft rather than with direction of growth.

fore limb put in place of a hind limb, fig. 28, Plate 12, becomes innervated by the lumbo-sacral plexus. Furthermore, as Hamburger (1928, 1929) has shown, the normal arrangement occurs, irrespective of the quantity or the quality of the nerves entering it, as well as of their origin (p. 183).

The following considerations may facilitate an understanding of this relation. The limb bud in the Amphibia is small and a mere nodule of mesenchyme when the first nerves reach it close to the blood vessels that precede them. At least some of the primary connections may be assumed to be made then, although the definitive nerve endings, according to observations upon embryos of birds and mammals (Tello, 1917; Cajal, 1919), are not developed until later. Subsequently the elongation of the bud takes place, and the individual muscles and cartilages segregate out of the original blastema. As this proceeds, the nerves are drawn out and shifted with the individual segregates and thus assume a normal arrangement within the appendage, whatever their origin may be.

The adjustment between ingrowing nerves and end organs, while exact as to final results, may accommodate itself within a wide range of time and space, as shown by the following examples.

When by an appropriate experiment a limb bud is made to undergo its early development in the absence of nerves and then is grafted to a normal embryo, the nerves of the latter form their connections in the limb later than they usually do, and yet they assume a normal arrangement (Harrison, 1907).* Between the two species, *Amblystoma punctatum* and *A. tigrinum*, there is a marked difference with respect to the relative time of growth and differentiation of the fore limb. When a tigrinum limb bud is grafted to a punctatum embryo, its relative growth is so slow at first that the limb is still but an elongated bud, fig. 24, *gr*, Plate 12, when the normal limb (*n*) of the host shows elbow joint and two long digits and has nerve trunks running to the hand. Yet, when the grafted limb finally reaches this condition, the nerves, which have grown in from the host, show normal distribution. In the reciprocal experiment the punctatum limb on the tigrinum host is precociously developed, fig. 23, *gr*, Plate 12, and the nerves are normally distributed days before the proper time. Sections taken at suitable stages show nerves reaching at least to the forearm of the grafted limb, while on the normal side they run merely to the base of the bud which is but a nodule of mesenchyme.

The adjustment of nerves to a different spatial scale is shown in regenerated limbs. In the salamander, *Eurycea*, Weiss and Walker

* Braus (1905), who originated this experiment, obtained different results and interpreted his findings in accordance with the protoplasmic bridge theory.

(1934) found that the nerves in regenerated limbs correspond in their arrangement precisely to the nerves of the primary limb, although the regenerated appendage develops from a mesenchymal blastema that is much larger than that of the embryonic limb bud. The nerve fibres are more numerous in the regenerated limb, but the individual units are of the same order of magnitude in both cases.

Attempts to obtain limbs without nerves have led to the discovery of some very interesting facts regarding the paths taken by fibres that do

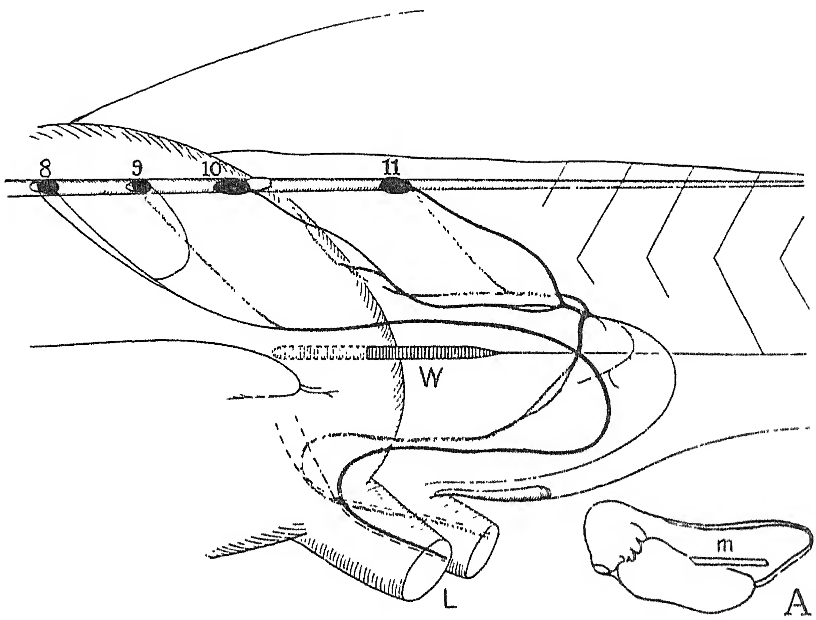


FIG. 30—Innervation of hind limbs of a frog (*R. temporaria*), in which the nerves were prevented from entering the limbs directly by the insertion of a mica plate (*m*) between notochord and limb rudiment of the embryo, shown in the small figure (A). The nerves 8–11 run around the posterior border of the scar (*W*), completely reversing their direction to reach the base of the limbs (*L*). After Hamburger (1929).

succeed in reaching their destination. To prevent their entrance into the hind limb in embryos of the frog or toad, a longitudinal horizontal incision is made through the posterior part of the trunk just below the notochord (Harrison, 1904; Hamburger, 1928, 1929). An object, best a thin plate of mica, is then thrust through the slit in order to keep the wound from healing completely, fig. 30 A. Even so it is difficult to prevent all nerves from reaching the limb, and out of seventeen cases investigated by Hamburger only four had the limbs on one or both sides entirely without nerves.

The nerveless limbs do not interest us particularly here. They are normally formed with joints and toes, and the tissues are differentiated, but they are markedly atrophic and functionless. The partly innervated limbs, on the other hand, are very important for our present purpose.

To reach the limbs, nerves may take the most circuitous courses. They may form a plexus across the mid line above the wound and curve as much as 180° around either the posterior or the anterior border to reach the limb, fig. 30. When one-half of the medullary cord in the region of the hind limb is excised, although a considerable amount of regeneration usually takes place across the median plane, there are some cases in which no nerves arise on the side of the operation. In some of these, nerves from the opposite side cross the mid line from the intact side and thereby reach the opposite limb, a course that no nerves normally ever take. Whether these facts are to be taken as showing that regions without nerves, particularly the growing limb bud, exert a positive directive influence upon developing nerve fibres, or whether they are better explained by assuming that the configuration of the interstitial tissue is such as to guide the nerves in their peculiar course around the wound, cannot be decided from the evidence at hand.

When the motor areas are eliminated by cutting out a median strip of the medullary plate, there is a tendency for the cord to reorganize itself and form some motor elements. However, one case was obtained by Hamburger in which only sensory nerves were present on both sides, with the exception of a small motor component on the right. While only two small branches to muscles, probably proprioceptors, were found, the plexus was normal and nerves were normally distributed in both legs, all of the sensory branches being present. Partial motor innervation, without sensory nerves, was found in one case, as a result of incomplete restitution of the motor part of the cord after excision of one lateral half. The motor nerves were distributed normally as far as they went. Both sciatic and crural nerves were present and the former could be followed to below the knee.*

Although sensory and motor nerves normally run together, the foregoing experiments show that either may find their way alone. In so doing the sensory nerves disregard possible short cuts to the skin, and follow the usual paths between the muscles.

* By excising the dorsal half of the cord in the limb region of *Amblystoma,* Detwiler and Vandyke (1934) have also obtained limbs with efferent nerves only. Their mode of action is of great interest, but as yet the exact distribution of the intrinsic branches has not been followed.

Hamburger finds that the cases in which the nerve trunks accompany the main arteries have much more complete innervation. When the nerves run alone, they tend to scatter in the proximal part of the limb, innervating only single muscles or restricted areas of skin. While there is no rigorous experimental proof that the nerves are actually attracted by the arteries, the blood vessels do precede the nerves in the developing limb bud (Tello, 1917). It has been suggested by Weiss (1934, p. 433) that the close contact between the two may be explained by the assumption that outgrowing nerves and vessels respond to a common agent, the ultrastructure of the interstitial ground substance.

One of the most baffling questions in the development of peripheral nerves is the selectivity of the fibres in establishing their proper terminations—motor neurones with muscle fibres and sensory neurones with the epithelium of the skin or mucous membranes or with muscle spindles. In all of the spinal nerves motor and sensory fibres join beyond the ganglia and run together, yet without confusion. The growing fibres connect with the proper end organs, where they form characteristic arborizations. There is even a strict selectivity among sensory nerves, as pointed out by Cajal (1919), in connection with the nerves of the tongue, where the trigeminus forms general sensory endings and the facial and the glossopharyngeal run to the taste buds. It seems necessary to assume some specific reaction between each kind of end organ and its nerve (Harrison, 1910), and Cajal (1919) and Tello (1923) have both pointed out that this could scarcely be of other than a chemical nature. However, there is no experimental evidence to prove the latter, and the experiments of Boeke (1913, 1914, 1917) in cross-suturing sensory and motor nerves render a satisfactory explanation on this basis very difficult. These experiments, which concern especially the lingual and the hypoglossal nerves in the hedgehog, show that regenerating fibres may be forced to form anomalous connections. Motor hypoglossal fibres, conducted through the peripheral stump to the mucous membrane, form there typical sensory end organs and may even stimulate the rehabilitation of taste buds. Similarly, regenerating sensory lingualis fibres, passing through the peripheral hypoglossal trunk, reach the muscles and form typical motor and plates. While the latter are almost exactly like normal end plates, the sensory end arborizations formed by the hypoglossal are frequently atypical and may show some influence of the quality of the regenerating nerve. Another more recent experiment by Boeke and Dijkstra (1932), in which skin was interchanged between leg and bill in the duck, shows likewise that nerve fibres are non-specific. The corpuscles of Grandry and of Herbst, found normally only in the bill, first

degenerate after transplantation, and then regenerate, becoming innervated by nerves of the leg. In skin from the leg grafted on the bill, no such sense organs are formed in spite of the presence of nerves normally innervating them.

The heterogeneous mass of facts referred to in this section cannot at the present time be brought under one rubric. However, many of them may be interpreted in the light of the stereotropic responses demonstrated by the tissue culture experiments. Most difficult to explain is the formation of terminal connections, and here, notwithstanding the inconsistencies that have been recorded, one can scarcely but agree with those who postulate a response to some sort of chemical stimulus.

Responses of the Nerve Centres to Changed Conditions at the Periphery and in other Parts of the Central Nervous System

After the first fibres are laid down, the nervous system passes into another phase of development, in which functional activity of some sort begins to play a role.

It has long been known that when the end organ or the peripheral end of a neurone is destroyed and fails to regenerate, atrophy of the nerve cell follows. In the embryo an analogous process may occur, but here it is rather a failure to develop than an actual atrophy of nerve units already differentiated.

Responses of Sensory Centres

In addition to sporadic observations on defective monsters, there is now much experimental evidence of hypoplastic development of nerve centres following peripheral injury. Early removal of the eye in amphibian embryos is followed by a marked reduction in the walls of the mid-brain on the opposite side, as shown by Steinitz (1906) and Dürken (1913). A similar reduction in the olfactory centres occurs as a consequence of removal of the nasal pit, and in the absence of the nose the fore-brain does not regenerate after excision (Burr, 1916). In the chick embryo, after removal of the wing rudiment, the spinal ganglia of the region are much reduced (Shorey, 1909).

The positive counterpart to this, the hypertrophy or hyperplasia of nerve centres in response to peripheral overloading or to increased stimuli from within, was first discovered by Detwiler (1920, 1923) in connection with the spinal ganglia innervating supernumerary grafted

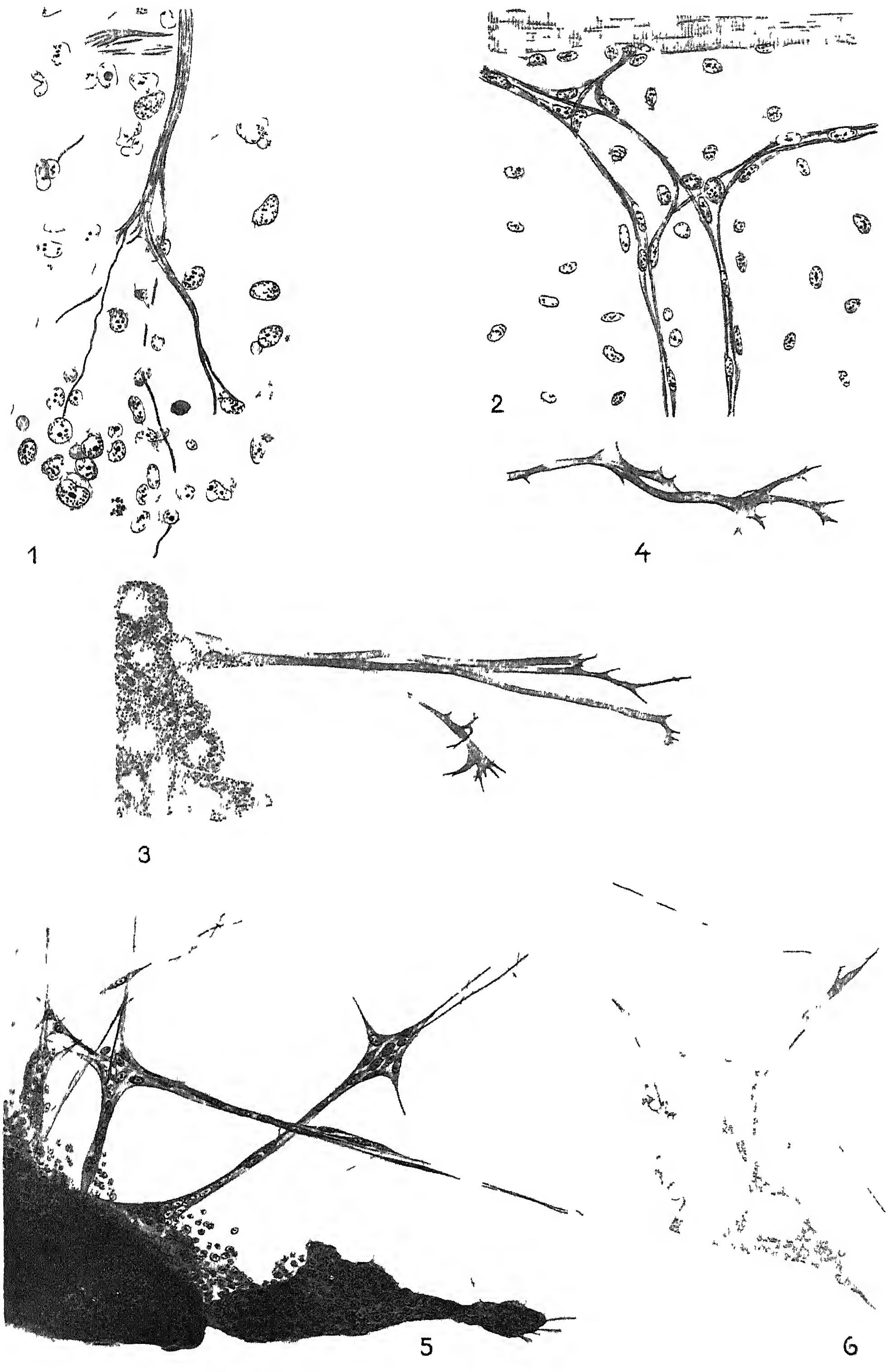
limbs. A limb bud, implanted on the flank in *Ambylstoma*, is supplied by nerves that ordinarily have a more limited distribution. Their ganglia become larger than normal and show an actual increase in cell number amounting to about 40%. The corresponding hypoplasia of the ganglia of the brachial plexus, after removal and non-regeneration of the limb bud, is about 50%.

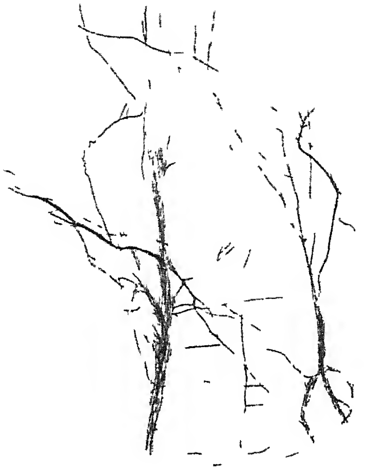
Experiments of the same general kind, but varied much in detail, have all confirmed these results. Limbs grafted to the head bring about hyperplasia of the cranial ganglia innervating them (Detwiler, 1930). Heteroplastically transplanted limbs that grow larger than the normal, fig. 22, Plate 12, have intrinsic nerves approximately proportional to their size, and the spinal ganglia supplying them usually show hyperplasia (Schwind, 1931), although the results here do not seem to be quite constant. Spinal ganglia give evidence of hyperplastic response to supernumerary limbs, grafted in the tail bud stage, as early as twelve days after operation, which corresponds closely to the time at which function begins in the limb,* and the capacity of the ganglia to respond to either augmentation or reduction of the peripheral field innervated by them persists until after metamorphosis (Carpenter, 1932, 1933).

Further cases of sensory hyperplasia have been obtained after transplantation of other organs, and, in fact, this phenomenon may be said to be of general occurrence after peripheral overloading. When a second nasal pit is grafted alongside the normal one and its olfactory nerve penetrates the telencephalon, hyperplasia of the latter ensues (Burr, 1930), and an analogous effect is obtained after implantation of a supernumerary eye (Pasquini, 1927).

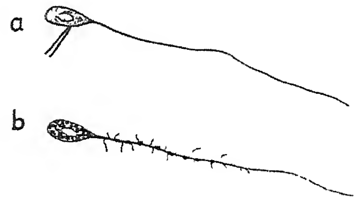
Perhaps the most nearly normal way to bring about overloading is by the method of heteroplastic grafting just referred to. The eye lends itself well to such experiments because of the ease and precision with which it may be measured in the living animal. The eye of *Amblystoma tigrinum* grafted upon *A. punctatum*, fig. 25, Plate 12, may attain twice the linear measurements of the normal eye of the latter species, the average ratio between the two eyes in such experiments being about 1 : 1·8 at the time of metamorphosis (Harrison, 1929). The retina of the large eye thus has from three to four times the area of the smaller one in the same animal, and although the number of cells in the ganglionic layer is less per unit area in the larger eye, the total number is much greater. Consequently, the optic nerve, which is derived directly from these cells, is larger, fig. 31, Plate 13. If it connects normally with the brain and

* Unpublished work by Muschenheim referred to by Detwiler (1933, p. 289).

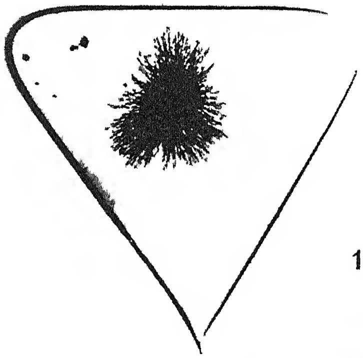

1
2
4
3
5
6



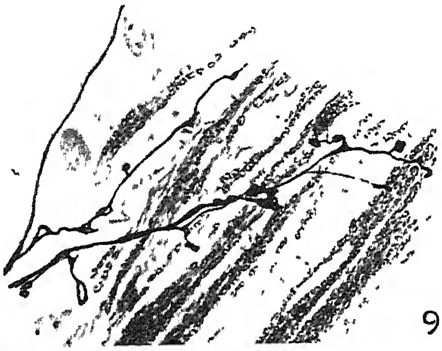
7



8

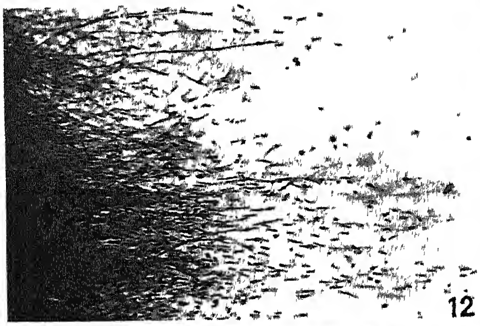


10

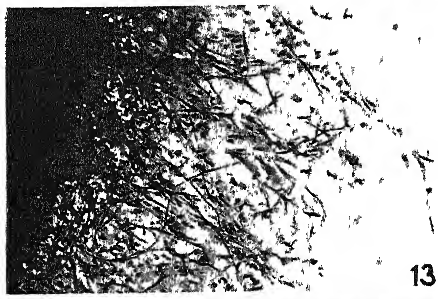


9

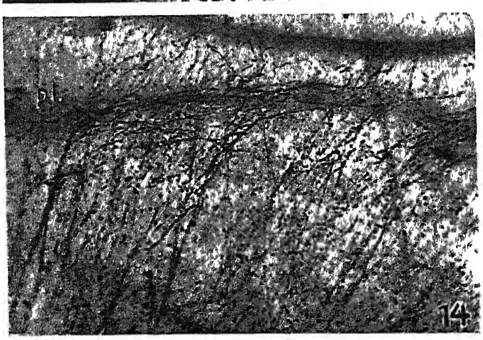
11



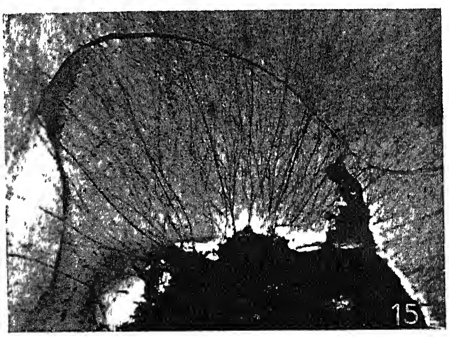
12



13



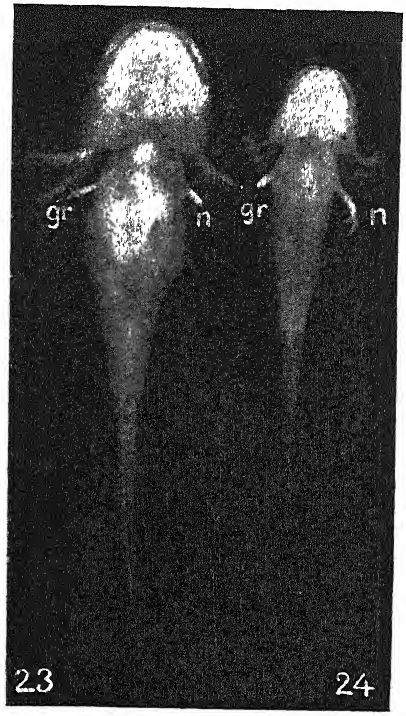
14



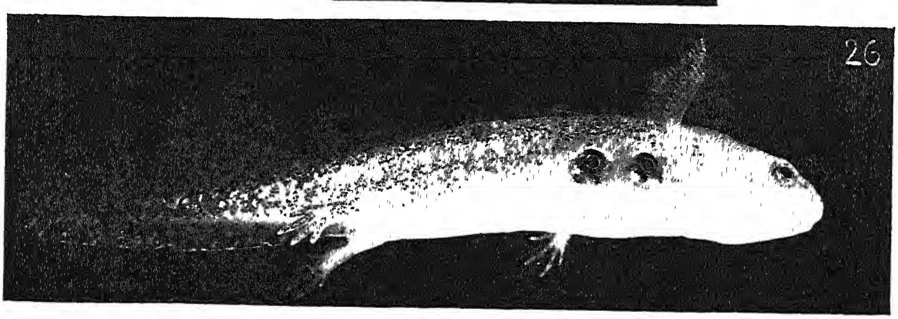
15

 Proc. Roy. Soc., *B*, *vol.* 118, *Plate* 12

22

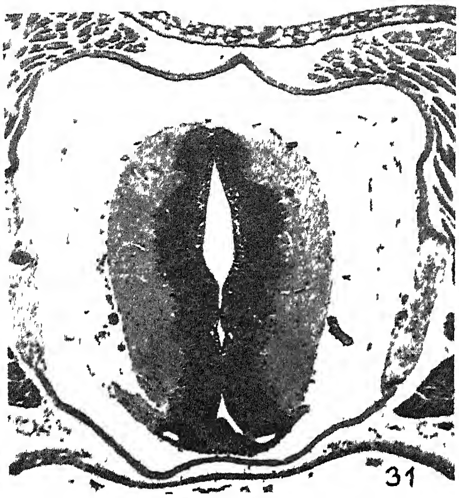


25

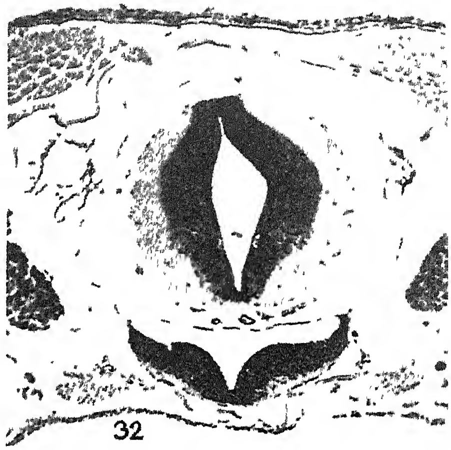


27

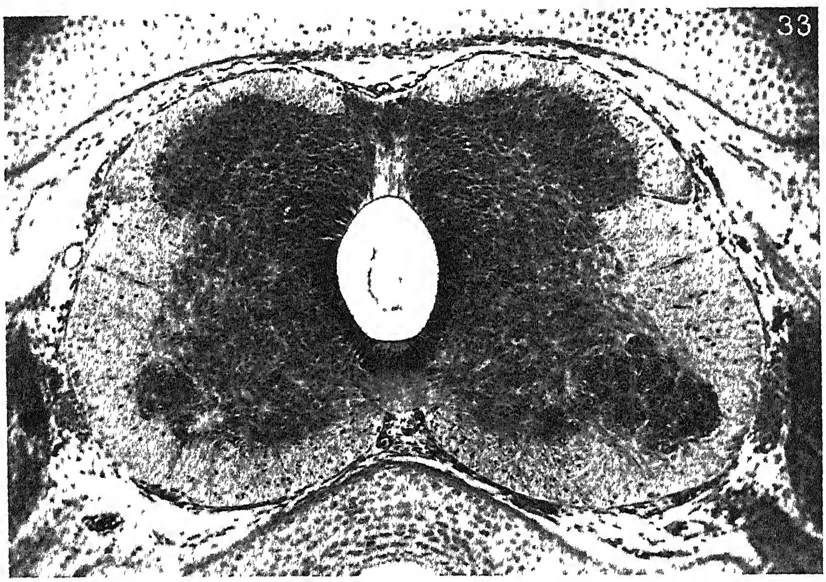
28



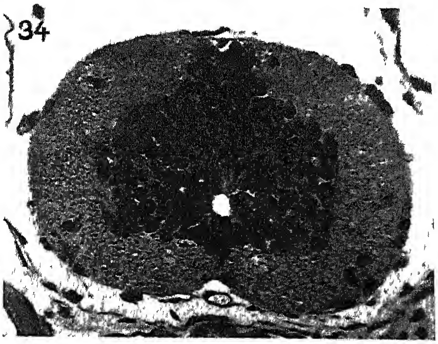
31



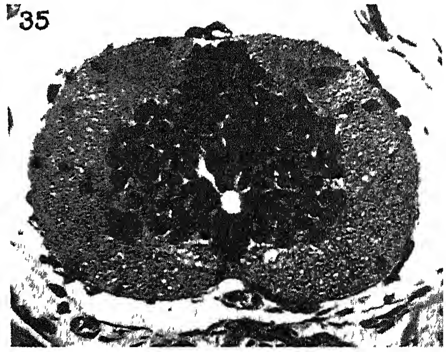
32



33



34



35

crosses to the opposite side, that side of the mid-brain undergoes hyperplasia and bulges markedly, fig. 32, Plate 13. Twitty (1932) has found that the number of cells in the grey substance may exceed the normal by more than 20%. Similar experiments with nasal pits (Burr, 1930) and with the ear (Richardson, 1932) give comparable results, though the amount of hyperplasia is apparently less.

In order to induce hyperplasia, it is not necessary that the peripheral overloading be of a nature to produce normal function. After implantation of a supernumerary nasal pit close to the normal one, the olfactory nerve of the former, instead of joining the latter, may run independently and enter the *pars dorsalis thalami*, in which an increase in the number of cells amounting to about 20% may then be observed (Burr, 1924, 1930). Sometimes olfactory fibres may join the ophthalmic nerve; if so its ganglion becomes larger. Analogous results were obtained by May and Detwiler (1925) after transplantation of the eye and the nasal pit to the region of the ear. The optic nerve then runs directly into the acoustic region of the medulla or into the vagus ganglion with a resultant hyperplasia that may reach 20%in the former and 50% in the latter.* That function in the ordinary sense is not concerned with this reaction follows also from experiments by Burr (1920) in which it was shown that even when the nasal pit is buried under the skin, so that stimuli cannot reach it from the outside, hypoplasia does not occur in the telencephalon, provided the olfactory fibres enter it.

These findings accord well with the results of earlier experiments in which frog embryos were reared in chloretone between the time of closure of the neural folds and complete resorption of the yolk, a period of about seven days, during which normally the primary nerve paths are all laid down and various co-ordinated movements initiated (Harrison, 1904). Embryos so treated remain motionless throughout and do not respond to tactile stimuli. Nevertheless, when finally placed in pure water at the expiration of the period, they exhibit perfect co-ordination in their movements as soon as the anaesthetic is eliminated; this may not require more than fifteen minutes, while normally the movements develop by gradual stages, correlated with the degree of differentiation of the nervous connections (Coghill).

The above facts all indicate that the factors influencing the development of co-ordinating mechanisms are not nervous stimuli of the usual kind. Thus even in these relatively late stages, the developmental functions remain distinct from the common physiological processes.

* *See also* May (1927).

That the aberrant olfactory nerves, mentioned above, usually enter the brain at a particular place in the thalamus, is correlated, according to Burr (1932), with the presence of a centre of active cellular proliferation at that point when the nerve approaches it. This is a reciprocal relation, for the ingrowing fibres, in turn, stimulate the cells of the centre to increased activity, leading to the observed hyperplasia. Something of this kind may take place in normal development, for, according to Coghill (1924), the distribution of dividing cells in the central nervous system of the embryo is inequal, with a series of zones of more intense activity alternating with zones of less, and Burr (1932) finds that the active regions correspond to the points of entry of the sensory roots of the cranial nerves.

Responses of Motor Centres

The foregoing observations and experiments, which have to do with afferent centres, are remarkably consistent. With regard to the motor centres the evidence is conflicting.

Shorey (1909) reported that when the limb bud or myotomes are excised, both motor and sensory nerves which normally run to them are defective, with a corresponding reduction in the ventral horns of the grey matter of the spinal cord as well as in the spinal ganglia. This was held to apply to urodele and anuran amphibians (*Amblystoma* and *Bufo*) and to the chick. With regard to the latter, Shorey's results have recently been confirmed by Hamburger (1934), who finds that after extirpation of the wing rudiment the hypoplasia of the lateral motor group of ventral horn cells ranges from 28 to 61%, fig. 33, Plate 13. May (1933) has found essentially the same in the anuran, *Discoglossus*, and maintains also that overloading by implantation of a supernumerary limb is followed by hyperplasia of the motor centres.

On the other hand, Detwiler's earlier and much diversified experiments upon *Amblystoma* have yielded invariably negative results. There is no reduction in the number of cells in the ventral (motor) sector of the spinal cord after extirpation of myotomes or limb buds and no hyperplasia after implantation of supernumerary appendages, figs. 34 and 35, Plate 13. Weiss (1931) likewise failed to find motor hyperplasia in an adult toad with supernumerary hind legs. There is, however, a small reduction in the total mass of the motor region of the cord, accompanying defects at the periphery, which is due to the smaller size of the motor cells, and this is greater when the defects (ablation of limbs) are bilateral (Detwiler and Lewis (1925)).

The stimuli to the motor cells from within the central nervous system constitute in the urodeles the principal factor regulating the number of these elements. This has been shown by Detwiler (1923–1933), by means of a series of experiments in which portions of the hind brain and spinal cord were removed and transplanted in various ways. Substitution of a piece of spinal cord, comprising the seventh, eighth, and ninth segments, for the brachial region (third, fourth, and fifth), which normally contains a larger number of motor neurones, is followed by hyperplasia of the motor cells of the graft to the extent that the normal number for the brachial region is attained. This means a hyperplasia of about 80%, and it takes place even after excision of the fore limb, which receives its innervation from that region, showing that it is independent of peripheral loading. If the corresponding segments are left in place and a limb is transplanted so that it becomes innervated by them, the hyperplasia does not occur. A similar effect is obtained after reversal of the brachial region of the cord, whereby the fifth segment, which normally contains fewer motor cells than the third, is placed, with polarity reversed, in the position of the latter and *vice versa*. The number of motor cells found then in each segment in the mature larva is the number proper to its new position. Independent positive evidence of the influence of centres higher up in the central nervous system has been obtained by substituting for the first five segments of the spinal cord a piece which included the posterior part of the medulla oblongata and the first two spinal segments. An appreciable hyperplasia occurred both in the two transplanted spinal segments and in the first two intact segments (sixth and seventh) posterior to the brachial region. This can be ascribed only to the influence of the increased number of neurones in the two medullae combined, which give rise to a larger than normal number of descending fibres (bulbo-spinal tract) in the cord.

If, on the other hand, the medulla is extirpated, there is a reduction in the number of cells in the first three spinal segments, which is the case only to a much less degree when the medulla is left intact and the mesencephalon removed (Nicholas, 1929, 1930). Also in the chick embryo isolation of the lumbar cord from the regions above and below is followed by hypoplasia (Williams, 1931).

At present there is no ready explanation of the conflicting observations regarding the response of motor centres to changes in conditions at the periphery. The most probable one is that they are due to real differences in the conditions obtaining in the species used. In the chick the motor nuclei are compact and well marked off from other centres, fig. 33, Plate 13, while in the urodele amphibians the motor cells are widely dispersed

and without definite limits. Inasmuch as hyperplasia follows stimulation which does not pass over the neurones that are themselves affected but over neighbouring ones (p. 191), Hamburger (1934) suggests that they may be too scattered to be influenced in the urodeles while readily affected in the chick. The anuran amphibians occupy an intermediate position. Whatever the ultimate solution of the discrepancies may be, it is well established that the motor centres are markedly influenced by central impulses, *i.e.*, by stimuli received from within.

That still other factors may play a part in regulating the proliferation of neuroblasts is shown by the experiments of Severinghaus (1930) in grafting segments of the spinal cord, together with notochord and myotomes, to the flank of another individual without direct connection with the central nervous system of the latter. In such cases cell proliferation runs wild, and in the dorsal (sensory) half of the cord the number of cells may reach three times the normal, as if some inhibitory factor had been removed.

Summary and Conclusion

Proof that a kind of functional regulation is active in determining the final relations of the elements in the nervous system must not be taken to imply that influences of this nature are the only factors involved or even the most important ones, for the material in which this regulation takes place has already acquired in advance most of its essential qualities. The protoplasmic characteristics of the species are already laid down in the egg and, being shared by the material of the presumptive brain and spinal cord, render the nervous system of one species different from that of any other. This prospective nervous material is, moreover, subject to the influence of the " organizer " underlying it in the gastrula, and in the absence of this substratum its potential qualities are not realized.

In the period of the medullary plate, which is less understood for lack of appropriate experiments, considerable plasticity still remains, the form of the central nervous system gradually becoming fixed under the continued action of the underlying layer. By the time of closure of the medullary folds its main topographic features are established, although the differentiation of nerve cells has not yet begun. Then follows a period of differentiation, during which the neuroblasts give rise to the first fibre tracts within the central nervous system and to the first peripheral nerves. The original direction of outgrowth seems to be governed by the polarity of the neuroblasts, *i.e.*, it depends primarily upon qualities inherent in the latter at that time, although the mode of establishment of

this polarity is still conjectural. The outgrowing fibres may divide or be swerved from their original course by the configuration of the embryonic organs and the membranes surrounding them, and are guided by the micellar structure of the interstitial ground substance. Beginning at this time and continuing for a considerable period thereafter, there is an active localized proliferation and differentiation of neuroblasts by which the principal nerve centres are segregated and their relations to the nerve tracts established. This period, as we have just seen, is characterized by a delicate adjustment of cell number to stimuli received from other regions of the embryo, both from the periphery and from the central nervous system itself.

In the last period influences affecting the neurones are probably in part growth regulating factors of a general nature, while others are of a kind approaching specific nervous stimuli but not yet identical with them. Even the mode of reaction to changes in the peripheral sensory field can hardly be exactly the same in all cases.

When a sensory area like the eye or nose is decreased by excision or augmented either by doubling or by means of heteroplastic grafting, the operation itself changes the prospective number of primary sensory neurones and consequently brings about an actual decrease or increase in the number of axones entering the central nervous system. It is not difficult to imagine how the altered number of nerve fibres, with their terminal arborizations, might influence cell division and differentiation in the centres with which they connect, although even here it is not a strictly nervous function but something more of the nature of trophic stimulus that produces the result (Burr, 1920). How the spinal ganglion cells become similarly affected by an alteration in the peripheral area supplied by them is another question, since here the primary neurones are not changed directly by the operation. One would naturally think that the outgrowing sensory fibres might accommodate themselves to the peripheral disturbance by an adjustment in the frequency of branching of their axones, as the motor fibres undoubtedly do. That this direct response at the periphery may occur to some extent is not disproved by any of the experiments, but the hypoplasia or hyperplasia of the ganglia, which is the more conspicuous response, is difficult to understand, for it is not the cells with processes reaching to the periphery that respond in this way, but the undifferentiated cells far off in the ganglia. The former must therefore carry impulses to the centre that stimulate neighbouring cells to send additional axones to the periphery.

With the motor neurones, the reaction to stimuli reaching them from within the central nervous system is again not so difficult to comprehend

and is probably of the same nature as the reaction of nerve centres to changes in the primary sensory neurones. Response of motor cells to peripheral influences must take place in urodeles largely by means of axonal division, as is shown by the difference in size between the proximal and distal portions of the nerves. On the other hand the central responses to such influences, reported by Shorey, Hamburger, and May in the anuran *Amphibia* and in birds, must be mediated in the same complicated way as those of the spinal ganglia.

The experiments last referred to deal almost entirely with the middle stages of development, in which regulatory responses of structure are obviously related to changes experimentally produced in other parts of the organism. As development proceeds, such responses become more and more difficult to follow, and yet many structural changes must take place in older animals, corresponding to the functional processes known as conditioning of reflexes, habit formation, and learning. Here again the traditional conflict arises between the treatment of the organism as a whole and the analytical method of approach by means of anatomical and physiological experiment with constituent parts. In Lashley's experiments on cerebral localization in the rat, the importance of a specific topographic arrangement of neurones seems to fade in comparison with that of some kind of representation of totality in every region. Nevertheless, the adjustments made to meet the loss of a large amount of cortex, which require considerable time to be effected, must have a structural basis and must result in a new configuration of the remaining material. Thus at the last stage of development of the organism we are still confronted, as at all previous stages, with this most significant but perplexing problem of biology: how an organism can be made up of independently working parts, and, at the same time, have the whole represented in each part.

In the new experiments that will be required to throw light on this field, embryology will need the assistance of the most delicate methods of modern physiology. In return it will be able to contribute materially to the advancement of physiology, whenever the latter is ready to make use of the methods which experimental embryology has developed. By ablation and transplantation of parts of the embryo it is possible to fashion almost any kind of nervous system desired and subsequently to study its function without the disturbing effects of trauma. This is one of the most promising lines of investigation leading off from the field covered in the present lecture.

References

References not listed below may be found in one of the four following papers:—
Detwiler, S. R. (1933). 'Biol. Rev.,' vol. **8**, p. 269.
Harrison, R. G. (1910). 'J. exp. Zool.,' vol. **9**, p. 787.
—— (1924). 'J. comp. Neurol.,' vol. **37**, p. 123.
Mangold, O. (1928). 'Ergebn. Biol.,' vol. **3**, p. 151.

Baitsell, G. A. (1917). 'Amer. J. Physiol.,' vol. **44**, p. 109.
Bauer, K. (1932). 'Z. mikr.-anat. Forsch.,' vol. **28**, p. 47.
Bethe, A., and Fischer, E. (1931). 'Handb. norm. u. pathol. Physiol.,' vol. **15**, p. 1045, Berlin, Springer.
Bisceglie, V., und Juhász-Schäffer, A. (1928). "Die Gewebezüchtung *in vitro*," Berlin, Springer.
Boeke, J. (1933). 'Zeit. mikr.-anat. Forsch.,' vol. **33**, pp. 23, 233.
Boeke, J., und Dijkstra, C. (1932). 'Proc. Akad. Sci. Amst.' vol. **35**, p. 1114.
Burr, H. S. (1932). 'J. comp. Neurol.,' vol. **56**, p. 347.
Cajal, S. R. y (1908). 'Anat. Anz.,' vol. **32**, p. 1. Reprinted in "Études sur la Neurogenèse de quelques Vertébrés," Madrid, 1929, pp. 81-125.
—— (1919). 'Trab. Lab. Invest. biol. Univ. Madr.,' vol. **17**, p. 181. Reprinted in "Études sur la Neurogenèse de quelques Vertébrés," Madrid, 1929, pp. 297-348.
—— (1928). "Degeneration and Regeneration of the Nervous System," translated from the Spanish edition (1913) and edited by R. M. May, vol. **1**, Oxford University Press.
Carrel, A. and Burrows, M. T. (1911). 'J. exp. Med.,' vol. **14**, p. 244.
Coghill, G. E. (1929). "Anatomy and the Problem of Behavior," Cambridge University Press.
Detwiler, S. R. (1932). 'J. comp. Neurol.,' vol. **56**, p. 465.
—— (1934). 'J. exp. Zool.,' vol. **67**, p. 395.
Detwiler, S. R., and Lewis, R. W. (1925). 'J. comp. Neurol.,' vol. **39**, p. 291.
Detwiler, S. R., and Vandyke, R. H. (1934). 'J. exp. Zool.,' vol. **68**, p. 321.
Esaki, S. (1929). 'C. R. Assoc. Anat.,' 24^{me} Réun., 13 pp.
Grigorjeff, L. M. (1931). 'Arch. exp. Zellforsch.,' vol. **11**, p. 483.
—— (1932). 'Arch. exp. Zellforsch.,' vol. **13**, p. 195.
Hamburger, V. (1934). 'J. exp. Zool.,' vol. **68**, p. 449.
Harrison, R. G. (1914). 'J. exp. Zool.,' vol. **17**, p. 521.
Herrick, C. J. (1924). "Neurological Foundations of Animal Behavior," New York, H. Holt & Co.
Hooker, D. (1930). 'J. exp. Zool.,' vol. **55**, p. 23.
Huzella, T. (1929). 'Roux Arch.,' vol. **116**, p. 430.
—— (1931). 'C. R. Soc. Biol., Paris,' vol. **106**, p. 179.
—— (1934). 'Arch. exp. Zellforsch.,' vol. **15**, p. 250.
Huzella, T., and Lengyel, J. (1932). 'Arch. exp. Zellforsch.,' vol. **13**, p. 141.
Kappers, C. U. A. (1908). 'Folia neuro-biol.,' vol. **1**, p. 157.
—— (1933). 'Psychiat. neurol. Bl. Amst.,' Jaarg., 1933, 25 pp.
Lashley, K. S. (1926). 'J. comp. Neurol.,' vol. **41**, p. 1.
—— (1929). "Brain Mechanisms and Intelligence," Univ. of Chicago Press.

Levi, G. (1925, *a*). 'Arch. exp. Zellforsch.,' vol. **1**, p. 1.
—— (1925, *b*). 'Arch. exp. Zellforsch.,' vol. **2**, p. 244.
—— (1934). 'Ergebn. Anat. Entw.-Gesch.,' vol. **31**, p. 125.
Lewis, W. H. (1907). 'Amer. J. Anat.,' vol. **6**, p. 461.
—— (1922). 'Anat. Rec.,' vol. **23**, p. 177.
Luther, W. (1934). 'Roux' Archiv.,' vol. **131**, p. 532.
Mall, F. P. (1893). 'J. Morph.,' vol. **8**, p. 415.
Matsumoto, S. (1918). 'J. exp. Zool.,' vol. **26**, p. 545.
May, R. M. (1933). 'Bull. Biol.,' vol. **67**, p. 327, Pl. 13-14.
Mihálik, P. (1932). 'Anat. Rec.,' vol. **54**, p. 149.
Mossa, S. (1927). 'Arch. exp. Zellforsch.,' vol. **4**, p. 447, Pl. 2.
Nageotte, J. (1927). 'C. R. Soc. Biol., Paris,' vol. **96**, pp. 172, 464, 828, 1268; vol. **97**, p. 559.
—— (1931). 'Ann. Anat. path. méd.-chir.,' vol. **8**, p. 1, Pl. 1.
Nicholas, J. S. (1933). 'J. comp. Neurol.,' vol. **57**, p. 253.
Olivo, O. M. (1927). 'Arch. exp. Zellforsch.,' vol. **4**, p. 43.
Péterfi, T., und Kapel, O. (1928). 'Arch. exp. Zellforsch.,' vol. **5**, p. 341, Pl. 29.
Péterfi, T., und Williams, S. C. (1933). 'Arch. exp. Zellforsch.,' vol. **12**, p. 210.
de Renyi, G. S. (1929, *a*). 'J. comp. Neurol.,' vol. **47**, p. 405.
—— (1929, *b*). 'J. comp. Neurol.,' vol. **48**, p. 441.
Richardson, D. (1932). 'J. exp. Zool.,' vol. **63**, p. 413.
Speidel, C. C. (1933). 'Amer. J. Anat.,' vol. **52**, p. 1.
Steinitz, E. (1906). 'Roux' Archiv.,' vol. **20**, p. 537.
Van Campenhout, E. (1930). 'J. exp. Zool.,' vol. **56**, p. 295.
Weiss, P. (1929). 'Roux' Arch.,' vol. **116**, p. 438.
—— (1931). 'Pflüger's Arch.,' vol. **228**, p. 486.
—— (1933). 'Amer. Nat.,' vol. **67**, p. 322.
—— (1934). 'J. exp. Zool.,' vol. **68**, p. 393.
Weiss, P., and Walker, R. (1934). 'Proc. Soc. exp. Biol. Med.,' vol. **31**, p 810.

Explanation of Plates

Plate 10

Fig. 1—Ventral ramus of a lumbar nerve of a larva of *Rana palustris* from which the ganglion crest was removed 7 days prior to the preservation of the specimen; no sheath cells are present, and the mesenchyme cells enter into no definite relation with the nerve fibres. Exp. Z65. × 350.

Fig. 2—Similar nerve from a normal larva (*R. sylvatica*), showing numerous sheath cells closely attached to the nerve fibres. Exp. Z77, control. × 350.

Fig. 3—Group of nerve fibres growing from a piece of medullary cord of a frog embryo (*R. palustris*) in clotted lymph, about 36 hours after explantation. Exp. Is135. × 350.

Fig. 4—End of a sensory nerve lying between the ectoderm and the muscle plates, from a parasagittal section of an embryo of *Rana pipiens*, 4 mm long. × 750.

Fig. 5—Piece of duodenum of a 9-day chick embryo cultivated 2 days in chicken serum supported by spider web. Note chains of cells closely applied to the web fibres. Exp. C48. × 300.

FIG. 6—Two cells from a preparation of the medullary cord of a frog embryo (*R. palustris*), growing in serum (from *R. clamitans*) and attached to spider web fibres, 8 days after explantation. Exp. SW39. × 300.

PLATE 11

FIG. 7—Plexus of nerve fibres growing on the coverglass in a plasma medium from a culture of rhombencephalon of a 77-hour chick embryo, 29 hours after explantation. After Levi (1917). × 350.

FIG. 8—Cell from the mesencephalon of a 7-day chick, grown 50 hours in plasma culture. *a*, resting cell touched by micro-needle; *b*, effect of stimulation shown in formation of coarse granules and fine protoplasmic processes on the fibre. After Levi (1925, *b*).

FIG. 9—Nerve fibres from a piece of brain of a 4-day chick embryo, cultivated 72 hours together with muscle fibres and showing end organs. Gold chloride preparation. After Grigorjeff (1932).

FIG. 10—Colony of fibroblasts from the heart of a chick embryo growing in a plasma film in a triangular frame. The greatest growth is perpendicular to the sides of the frame. After Weiss (1929). × 7.

FIG. 11—A straight tract of spindle cells and nerve fibres growing between two spinal ganglia in an extremely thin plasma membrane. Ganglia from a 13-day chick embryo, 3 days in plasma. After Weiss (1934). × 37.

FIG. 12—From a culture of spinal ganglion of a 13-day chick, 3 days in plasma. Cells and fibres oriented in one direction, corresponding to the stretching of the clot. Photograph by Dr. Paul Weiss. × *ca*. 75.

FIG. 13—Same culture as in fig. 12, taken from a part of the clot not under tension. Cells and nerve fibres not definitely oriented. Photograph by Dr. Paul Weiss × *ca*. 75.

FIG. 14—Fragment of medulla (6-day embryo), grown for 2 days in diluted plasma surrounded by more concentrated plasma. A nerve plexus (*pl*) has developed along the boundary between the two media. After Weiss (1934). × 136.

FIG. 15—Formation of plexus by nerve fibres (from the medulla of a 6-day chick embryo) grown for 2 days next to the surface of the clot, the culture exhibiting slight liquefaction. After Weiss (1934). × 52.

PLATE 12

FIG. 22—Fully-grown larva of *Amblystoma punctatum*, with large grafted right limb from *A. tigrinum*, 75 days after operation. Exp. NE19. × 1·75.

FIGS. 23 and 24—Reciprocal pair in which the right anterior limbs were interchanged; preserved 18 days after operation. Exp. NE133. × 4·5.

FIG. 23—*Amblystoma punctatum*. Grafted limb (*gr*) much further advanced than the normal (*n*).

FIG. 24—*A. tigrinum*. Grafted limb (*gr*) much less developed than the normal (*n*).

FIG. 25—Larva of *A. punctatum* with large grafted right eye from *A. tigrinum*, 11 days after operation. (*Cf.* figs. 31 and 32.) Exp. Oc20. × 1·75.

FIG. 26—Larva of *A. punctatum* with two eyes grafted on the side, the anterior one replacing the fore limb. *Cf.* fig. 29 for the arrangement of the spinal nerves in a similar specimen. After Detwiler and Van Dyke (1934). × 3·7.

FIG. 27—Young toad (*Bombinator*) about completing metamorphosis with fore leg grafted to side of head. After Braus (1908).

FIG. 28—Young toad (*Bombinator*), with fore leg in place of hind leg on the left side. After Braus (1908).

PLATE 13

FIG. 31—Cross-section through the diencephalon of a larva of *A. punctatum*, 41·5 mm long, showing the inequality of the two optic nerves, the one on the right (left of figure) coming from a large grafted axolotl eye. Preserved 102 days after operation. Exp. Oc23. × 35.

FIG. 32—Cross-section through the midbrain of similar specimen with an *A. tigrinum* eye. The wall of the left side (right of figure), which receives the larger optic nerve, is thicker and more bulged than that of the right. Specimen preserved 90 days after operation. Exp. Oc10. × 35.

FIG. 33—Cross-section of spinal cord in the region of the fifteenth ganglion of a chick embryo, 8 days after beginning of incubation, and 5 days after the removal of the ring wing rudiment. The right side of the cord (left of figure) is smaller. The hypoplasia is most marked in the lateral part of the ventral horn, and is less in the dorsal horn. There is no significant hypoplasia of the medial portions. After Hamburger (1934). × 113.

FIG. 34—Section through spinal cord of an *Amblystoma* larva from which the seventh, eighth, and ninth somites had been entirely removed on the right side in the tail bud stage. Cord essentially symmetrical and no significant difference between the two sides in the number of cells. After Detwiler (1929). × 260.

FIG. 35—Another section from the same individual.

581 9:914 2

Discussion on the Origin and Relationship of the British Flora

(*Held on March* 28, 1935)

I—The Scope of the Discussion

Professor Seward, in his introductory remarks, referred to the publication in 1899 of Mr. Clement Reid's " Origin of the British Flora," and spoke of the more recent researches of Mrs. Reid which have considerably extended our knowledge of British Tertiary floras. He drew attention to the striking contrast between the early Tertiary floras and the present plant population of Britain, and to the changes exhibited by successive floras in geographical relationship and in composition. He emphasized the importance of reconstructing the geological background as well as the botanical features of the series of floras preceding and subsequent to the Glacial period. Reference was made to the more controversial question of the effect of the Ice Age upon the plant-world, and he expressed the hope that the discussion would enable botanists to form a more definite opinion on the crucial problem of survival or extinction of flowering plants when the British area was exposed to the maximum invasion of ice.

Professor Seward offered a hearty welcome to Dr. du Reitz of Uppsala, whose work on Scandinavian floras provided particularly important data having a direct bearing upon the history of the Pre-Glacial and Post-Glacial floras of Britain.

II—British Floras Antecedent to the Great Ice Age

Mrs. E. M. Reid—It is right that a discussion of the Origin and Relationships of the British flora should include the Tertiary history of plants in Britain, because this history shows that the origin lies in a very remote past; and that the relationships have been developing throughout the whole subsequent period.

In considering the history I shall be obliged to bring my evidence not only from our own country, but from neighbouring parts of the Continent, because much of it is not to be found in Britain. Between the Middle Oligocene of Hampshire and the Upper Pliocene of Cromer there is a

long gap in British floral history embracing the Upper Oligocene, all the Miocene, and almost the whole Pliocene. This gap can only be filled in from neighbouring countries. But from the beginning of the Eocene till the Middle Oligocene the history is fairly continuous; though much the greater part remains to be deciphered.

In regard to our own study of Tertiary floras it has been a circumstance of the utmost importance that the order of study has been from present to past; known to unknown. Always there has been an element of the known to point the way through the strange assemblage which each new flora presents. In order of study came the Late Pliocene of Cromer, the Middle Pliocene of Tegelen, and the Lower Pliocene of Reuver; the two latter places being in the Netherlands. These three floras gave the clues which led to the interpretation of earlier ones. The clues were twofold. First was the discovery that whereas the Cromer flora was composed almost entirely of living British species, the Tegelen flora contained a number of exotic species; mostly plants now endemic to China and Japan. That was the first clue, used to decipher the earlier flora of Reuver. Here was found the second clue. Not only were there Chinese and Japanese genera and species, but the proportion of them was greater, and they were of a warmer type; besides, there was a marked occurrence of endemic North American genera and species, and of genera which in the present day form the well-known and remarkable link between the living flora of China and Japan on the one side and of Eastern North America on the other. That was the second clue. The Pliocene floras of West Europe evidently represented an extinct latitudinal link between the two living floras.

To explain survival in the two regions and extinction in the third an old theory of migration was adopted and elaborated, namely, that a succession of ever cooler circumpolar floras was driven southwards by the cooling of the polar regions; that whereas in Eurasia the plants were trapped and exterminated against the latitudinal barrier of mountains which arose in the Middle Tertiaries, in East Asia and Eastern North America the north-south trend of the mountain chains not only permitted north and south migration, but also movement in altitude as the climate changed.

By the application of these two clues—that the allies of Tertiary floras of West Europe were to be sought among the living floras of East Asia and North America, especially among the mountain floras; and that the older the flora the greater would be the number of these exotics, and the warmer the type—it became possible to decipher older Tertiary floras: the clues were found to be fairly trustworthy guides.

At this point came the study of the London Clay flora, notably the fruits and seeds strewn on the Sheppey beach. It brought us to a critical period, not only in Tertiary floral history but also in our own studies. The little that was known with certainty, and the much greater bulk of unsupported statement, suggested a very warm flora, probably tropical, having affinities with all the tropical floras of the world. To meet its probable requirements we began to study whatever fruits and seeds were available from tropical and sub-tropical plants of the whole world. But the examination of the fossils themselves, carried on simultaneously, quickly showed that it was not a flora of general tropical affinity but one predominantly of Indo-Malayan type, having its closest affinity with the plants of the Malayan Islands. Of the determined genera 73% have living relations in the Malay Islands. There follow in succession the Malay Peninsula, Further India, India and Ceylon, South China and Burma, the latter with 53%, Australasia with 40%, and Tropical Africa with 39%, America only 20%. These facts could not be explained by the hypothesis of southward migration. For how could a tropical flora have reached Britain from the north? If it did, what could the climate of Britain have been when the flora lived to the north; and still more what the climate of the present-day tropics? So far as the London Clay was concerned, southward migration had to be given up.

The problem of warm or hot floras in what are now cooler regions is not new. Indeed, it formed part of a subject of discussion in this Society but a few years ago, and many explanations have been offered. Bearing in mind the botanical facts, we examined these explanations, namely: transport by water from a distant tropical forest; adaptation to change of climate; unreliability of fossil evidence; Wegener's hypothesis of continental drift entailing changes in the position of the Poles and of the continents; Dr. Brooks's theory of changes in the distribution of land and water, especially in relation to the Tethys Sea, and of orographic changes. It was found that the last explanation alone could reconcile past and present plant distribution. Wegener's hypothesis led to the impossible situation that as the Atlantic Ocean grew wider the alliance between the floras of Europe and North America grew closer. Moreover, Dr. Brooks's hypothesis alone could account for the Indo-Malayan character of the London Clay flora. For, as the Tethys Sea must have brought the warm waters of the Indian equatorial current to our own shores and those of the Arctic Ocean, so the shores of that great sea could have offered a coastal pathway for the plants—*Nipa*, *Dracontomelon*, *Endiandra*, *Toona*, and the rest. The explanation, whilst it presents some difficulties, seems to offer a sound basis for further research.

The London Clay brings us to the earliest period to which in late years the history of the British flora has been traced. It is difficult to know in what degree the previous studies of earlier British floras are reliable, for they depend with but few exceptions on bare statements unsupported by evidence. The Continental evidence is stronger and is documented. If we may accept the main trend of the findings as correct, then the London Clay flora was preceded by one of cooler type having more generic affinity with the living British flora. If so, this would point to a hot flora accompanying a warming climate, and replacing a cooler type of flora. The fact that many genera now European, such as *Quercus*, *Fagus*, *Viburnum*, *Hedera*, which were then ousted, returned later as the climate cooled, suggests that they must have saved themselves by northward migration, or ascent of mountains. Into the history of these earlier floras we have not penetrated. They are mentioned merely to show in what a very remote past we have to seek for the beginnings of our plant population.

For the purpose of this discussion we may well take the London Clay as our starting-point; for in the London Clay only 2, out of the 70 genera whose relationships were determined, are related to genera now living in Britain; one to *Tilia*, the lime; the other to *Cornus*, the dogwood. And both genera are extinct. Therefore the peopling of Britain by its present flora may for present purposes be regarded as beginning in the London Clay. What happened between the London Clay in the Lower Eocene and the Hordle period at the top of the Eocene we are not yet in a position fully to know. Certain very valuable evidence has been obtained by Miss Bandulska from the Middle Eocene beds of Bournemouth which, though newer than the London Clay, belong to a period when the physiographical conditions were much the same. Consequently, as might have been expected, her evidence points to floral relationships very similar to those found by us in the London Clay. Miss Chandler is now engaged in further filling in this gap by extensive researches on material from the magnificent coast sections between Bournemouth and Hordle. But the fruits and seeds are all very small, sometimes extremely small, so that the work is slow and laborious.

Certain it is that by the time of the Hordle flora, when the Tethys Sea no longer communicated with the Indian Ocean, a great change had occurred. The Malayan element had almost gone; while at the cooler end of the floral range out of 35 determined genera 8 are British, and 4 others South European. The change thus initiated continued throughout the remainder of the Tertiary period. In the Middle Oligocene of Bembridge out of 35 genera 13 are British and 5 others South European. And it is to be

noted that in the Hordle flora only 3, and in the Bembridge flora 2, of those whose relationship was determined are extinct; whereas in the London Clay out of the 70 genera whose relationships were determined, 40 were extinct. The percentage proportions of British genera in certain Tertiary floras have been found as follows: Lower Eocene 2%, Upper Eocene 23%, Lower Oligocene 34%, Mio Pliocene 59%, Lower Pliocene 53%, Middle Pliocene 78%, Upper Pliocene 97%. Thus the gradual Europeanizing of the British flora is shown by the relationships first of families, then of genera, and lastly of species. First are found a few genera of British relationship; but they are extinct; the family is represented. Among the Hordle genera nearly a quarter are British; and they are living; but the species are extinct. So far as my own experience goes I first met with living species in the Mio Pliocene of France, and four of those species happen to be British—*Najas marina*, *Polygonum Convolvulus*, *Solanum Dulcamara*, and *Hyoscyamus niger*—making about 12% of the species determined. From that time onward living species occur in greater and greater abundance; and more and more are British. In the Reuverian about 18% of determined species; in the Teglian 70%; in the Cromerian 98%. The transformation was complete. Save for a few species the exotic element had gone. The living British flora had taken its place. With its further history this paper is not concerned.

But what does the Tertiary history teach us? In the first place at the beginning and, again, at the end of our period it is plain that plant life reacted to change of climate by migration, associated on the one hand with survival, on the other with extermination, either local, or complete. In the intermediate Middle Tertiary period, as shown by the percentages of British genera quoted above and by the studies of the Oligocene and Miocene on the Continent, the absence of any extreme changes of climate such as marked the beginning and end of the period is reflected in a more stabilized flora.

We are led to the conclusions that for the beginning of any plant population we must look to a very remote past; and for its immediate aspect to the opportunities that may have occurred during the subsequent period for immigration and survival.

There is another influence which has tacitly been assumed, but of which, beyond its existence, Tertiary palaeobotany knows nothing. I refer to the appearance of new forms of life. We find them—new genera, new species—replacing old forms. We can trace *Magnolia*, *Cinnamomum*, *Vitis*, *Rubus*, and many others through changing forms from a far bygone past to the present day. But how, when, and where the changes happened we do not know. The nearest we, in our work, have ever got

to the history of such changes is in Miss Chandler's study of *Stratiotes*, the water soldier. The European history of this ancient genus, now monotypic and confined to Europe, was traced with a fair degree of continuity from the Hordle beds to the present day. We now know it from the yet older Bracklesham beds of Hengistbury and Mudeford. We can see that it changed. But how, where, and when lie beyond the discernment of Tertiary palaeobotany.

Thus Tertiary history shows that the plant population of any given time and place is due to the reactions of change of climate upon plant life, whether upon old or new forms. Change of climate on the one hand causes destruction; on the other induces migration when physiographical conditions permit, and so results in survival. It is to these causes acting throughout the ages that is due the establishment, at the time of the Cromer Forest bed, of a flora very similar to that of Britain at the present day.

III—The Quaternary Ice Age

(*a*) Professor P. G. H. Boswell, F.R.S.—*Climatic and Geographical Conditions in Britain during the Pleistocene Ice Age.*

The contribution of a geologist to a discussion on the Origin and Relationships of the British Flora can best take the form of an attempt to give word-pictures of the changing geographical and climatic conditions of the British area from late Pliocene to Post-Glacial (Post-Pleistocene) times. Any such attempt is at once attended with certain difficulty because the British Isles (at least in the south) were at one time part of an area wherein ice-margins oscillated as the glaciers advanced and retreated in successive phases of the Great Ice Age. As a result the features observable by geologists are complicated by the following considerations: (*a*) the beds deposited are heterogeneous and of rapidly varying lithology; (*b*) these beds were subjected to great erosion by the water from melting ice, consequently any soil or vegetation which had become established was the first to disappear; (*c*) the deposits are likely to have suffered repeated disturbance by successive ice-advances; and (*d*) in areas which have been subjected to more than one ice-advance, it is difficult, if not impossible, to say whether or not an area which became a plant refuge has persisted as an ice-free nunatak since Pre-Glacial times.

Evidence of not less than four, and possibly as many as six or seven, Pleistocene ice-advances has now been recognized in Britain. These glacial episodes were separated from one another by long or short periods

when the ice retreated as a result of greater or less amelioration. From the distribution of animal remains and of flint implements, we may conclude that the areas occupied by animals and man were restricted or extended as the ice advanced or retreated. The distribution of plant life is likely to have been similarly affected. The extents of the several ice-advances were marked by the limits of deposition of boulder clays, while the retreat-stages were accompanied by the production of water-deposited sands, gravels, silts, and bedded clays. The latter group constitute the interglacial deposits, which contain in places beds of peat or layers of leaf- and seed-bearing clays.

Beginning with the latest Pliocene deposits (in the sense in which the term Pliocene is at present used in Britain) which directly underlie the earliest Pleistocene (or Glacial) beds, I must refer to two plant-bearing horizons: (*a*) the Cromer Forest bed, and (*b*) the Castle Eden bed on the Durham coast. The fauna of the former consists of terrestrial, freshwater, and marine species. The terrestrial fauna includes predominantly warmth-loving animals, the teeth and bones of which may have been carried to their present situation from the south by the great river of Forest-bed times. But a few tundra species are also present; such as the southern mammoth, musk-ox, and a fossil elk. It is worthy of note, also, that the marine mollusca of the deposit are cold-water forms. The flora resembles that of the Cromer area at the present day (C. Reid), but, as Miss Chandler points out, it includes a few warm southern European elements. The Castle Eden flora is found in a deposit of clay which was torn up from the present area of the North Sea during the oldest glaciation of the East Coast (that which formed the Scandinavian Drift of West Hartlepool, discovered by Dr. C. T. Trechmann). The flora was referred by Mrs. E. M. Reid to the Middle Pliocene, for the percentage of exotic and extinct species (including Chinese and North American elements) was considerably higher than in either the Tegelian of Holland or the Cromer Forest bed deposits.

The beginning of Pleistocene times was marked in the east and south of England by a great marine transgression; and in this sea were deposited the sands and gravels known as the Westleton beds, extending from Norfolk to the London Basin, and possibly over much of the Midlands and south-west of England. Into this area debouched ice-streams bearing Scandinavian erratics; and the melting of these ice-streams produced the boulder clays known as the Norwich Brickearth, the Cromer Till, and the Scandinavian Drift of the Yorkshire and Durham coasts. There is reason for thinking that icebergs rather than ice-sheets were just able to reach the British coasts. The submergence of the land was such that

there was not likely to be land-connection with the Continent at the time. Certain high-level plateau drifts, like those described by Dr. K. S. Sandford occurring near Oxford up to 600 feet above O.D. and containing Scandinavian rocks among other erratics, appear to belong to this stage.

During the succeeding (and first) interglacial phase, the Scandinavian ice-sheet retreated and the east of England became a lake-like area in which were laid down sands and gravels distinguishable only with difficulty from the Westleton beds. The only land deposits preserved are high-level terraces, and no flora is known. Indeed, there would not appear to have been any great amelioration of climate, for the scanty evidence available indicates the preservation at a few localities of a cold-water marine shelly fauna (*e.g.*, at Billockby, near Great Yarmouth), as described by F. W. Harmer. The second glaciation was characterized by the growth of ice-sheets on the higher grounds of Britain, the glaciers spreading out fan-wise in their southward movement as far eastwards as Great Yarmouth, as far southwards as Finchley, and as far south-westwards as Moreton-in-Marsh. The product of this ice-advance was the Great Chalky Boulder Clay, a deposit which became Chalky-Neocomian, Chalky-Jurassic, or Chalky-Triassic, varying from east to west according to the outcrops of the solid rocks traversed by the ice. The remarkable Cromer Ridge, one of the most striking scenic features of the east of England, probably formed one of the terminal moraines of the ice-sheet. No direct evidence can be adduced of land connection with Europe, but on indirect reasoning it is likely that, notwithstanding isostatic lag, the weight of the ice-sheet kept the British land-mass submerged. Investigators in the west and north-west of Britain have found traces of ancient boulder clay (as, for example, at Silloth and Maryport) which may also be referable to this glacial epoch. Extra-glacial deposits of the same approximate date (though perhaps belonging to the end of the phase) appear to be represented by the high-level terraces of the valleys of the Avon, Severn, and Thames.

In the (second) Inter-Glacial period which followed, the faunal and floral evidence indicate that climatic conditions in Britain south of the River Humber were generally similar to those at the present time. The deposits consist of sands, gravels, loams, and clays, with peaty layers, but unfortunately only a few basin-like areas have been preserved. They are worthy, however, of being mentioned severally. First in importance is the lake-like area of Hoxne, near the county boundary of Norfolk and Suffolk. Here both plants and animals indicate marked amelioration of climate, but with at least one cold episode. Early man was able to establish working sites, referable to Acheulian and early "Mousterian"

industries. The temperate beds (with leaves, seeds, and pollen) have revealed the existence of a flora similar to that of the surrounding country to-day, but perhaps more heavily forested with alder, oak, birch, pine, hazel, and spruce, and supporting numerous herbivorous animals such as horse and deer. Similar evidence, though less complete, is afforded by the ancient lake-basin of Hitchin, described by the late Clement Reid, whose insight anticipated the results of later investigations. He saw in the flora of that locality evidence of sylvan temperate conditions, with the land standing at a higher level than now.

At Clacton, in what is apparently an old river valley belonging to the Thames drainage, peaty clays have been found by S. H. Warren in association with beds containing the early flake implements of the industry known as Clactonian. The flora was of a dry, temperate type, not appreciably different from that of the neighbourhood at the present day.

Lastly, the long-famous plant beds of West Wittering in Sussex should be mentioned. Although their stratigraphical position is uncertain, they lie near sea-level on the south coast—a level which we associate with the presence of early flake implements (formerly grouped under the general term " Early Mousterian ")—and contain the freshwater shell *Corbicula fluminalis*. Thus they appear to belong to the second Inter-Glacial phase.

A series of deposits of doubtful age should now be mentioned. They comprise the boulder clays, sands, gravels, and peaty loams which occupy an ancient channel or fiord at Kirmington in northern Lincolnshire. The brown or purple boulder clays which underlie and overlie the plant-bearing beds have not yet been correlated definitely with boulder clays occurring farther south in England. The work of Mr. J. P. T. Burchell on the human industries of these deposits suggests that the boulder clay at the base of the deposits is the time-equivalent of the Great Chalky Boulder Clay, in which case the plant-bearing beds would correspond to some of those in the Hoxne section. As regards the plants, Clement Reid noted that, although the list of species was small, the flora indicated estuarine conditions in a sub-arctic climate. With one exception, the plants are still to be found in the neighbourhood of the Humber; but one of them, *Carex incurva*, is a sea-coast sedge not now ranging south of Holy Island.

Summing up, therefore, we have evidence that during the second Inter-Glacial epoch the low ground supported a rich fauna and flora, the geography being that of a country of woodland and meres, with abundant and full streams; but we have no knowledge as to whether the mountain-masses were free of ice. The evidence of channels below present river-levels and of the return of *Corbicula fluminalis* to many localities in the

British area, suggests that the land was higher and therefore communication with the Continent was established.

A re-advance of the ice—the third glacial phase—brought more chalky boulder clay and associated materials (known collectively as the Upper Chalky Drift) only so far south as near the Suffolk-Essex boundary, although it extended farther down some of the chief valleys. In the west of England and Wales glaciation was more extensive, for the main advances from the Lake District, the Pennines, and Wales dated from this time. The probable limit of the ice-sheets ran by way of the Ironbridge gorge in Shropshire southwards to the British Channel, into which Welsh ice poured, as well as Irish Sea ice which impinged on the low ground of Cardiganshire and Pembrokeshire. In the southern Midlands were the swollen representatives of such rivers as the Avon, Stour, Severn, and Thames, evidence of climatic and physical conditions being left in the form of warp, frozen soil, terraces of medium height and channelling of older deposits (Wills, Sandford, Tomlinson).

Retreat of the ice again set in, but our knowledge of the climatic and geographical conditions of the third Inter-Glacial phase is scanty. The Kirmington plant and shell beds already mentioned may belong to this phase. Farther south the geography of Britain was being shaped into its present general form. River terraces at relatively low levels and brickearths on valley slopes, containing such human industries as the Aurignacian, seem to have been formed at this time. In Yorkshire and the Lake District the retreat was perhaps not very considerable; indeed, ice-caps may have remained on the high ground. No plant-bearing beds referable with certainty to the third Inter-Glacial phase are known. It seems reasonable to suppose that Britain still formed part of the Continent, but the presence of a raised beach at Morston, on the Norfolk coast, at 25 feet above O.D., found by Dr. J. D. Solomon, throws some doubt upon the matter.

The ice which had retreated from the low ground around the Irish Sea, where it had left the Lower Boulder Clay of the Cheshire Basin, with a covering of sands and gravels, re-advanced in the fourth glacial episode. The main Lake District glaciation dates from this time; glaciers flowed across the Tyne gap to the East Coast, and down the East Coast from the southern uplands, bringing Cheviot and Scottish erratics by way of Hessle in Yorkshire as far as Hunstanton and Holkham in Norfolk. So distinctive is the petrology of the Brown Boulder Clay it left behind that if the ice had extended into the Trent Valley or farther south than the Norfolk coast, its debris would assuredly have been recognized. Wales was again glaciated as far as the low ground in the south of the principality.

Dr. A. Raistrick has adduced evidence to show that during this glaciation (or during the third or fourth glaciations) refuge areas of nunatak character existed in the Pennine area in northern Yorkshire and southern Durham; but no such nunataks east or west of the Eden Valley could be recognized by Dr. Trotter or by Dr. Hollingworth. Nor can I point to any from my experience in the Welsh mountains.

This fourth glacial episode may also have produced the beds with a cold flora (arctic birch and willow) of Barnwell Station, Cambridgeshire, and Ponders End in the Lea Valley. While it is tempting to make such a correlation, the stratigraphical evidence is perhaps hardly sufficient to justify it.

Subsequent oscillations of climate, although they caused, firstly, the retreat of the ice in Wales, Scotland, the Lake District, and the Pennines, and then a Scottish re-advance into the Lake District, again a retreat, and still later, the advances of corrie glaciers in the mountainous areas, did not lead to any material change in the geography of southern Britain. The country probably remained for some time united to the Continent. In very general terms it would appear that the last considerable subsidence, which drowned river valleys and produced submerged forests, causing important aggradation, was Post-Neolithic in age.

No mention has been made of Ireland in the above summary of events in the Pleistocene. Like Scotland, Ireland appears to have suffered at least two major glaciations, probably the last two. Whether or not there were earlier glaciations, of which the evidence has been obliterated, is uncertain. It is noteworthy that the existence of older Palaeolithic Man in Scotland and Ireland has never been established, while evidence of newer Palaeolithic Man is rare and a matter of controversy.

From the above account it is obvious that the older published maps which show the maximum glaciation of Britain as extending to near London and the south coast of Wales, do not furnish a true picture of events. Such a maximum limit is composite. In the four major glaciations of the British area the centres of dispersal moved south-westwards, and the area affected extended farther southwards, as the Atlantic Ocean was approached. The advance of knowledge in the last quarter of a century has been so considerable as to call for a new text-book on the Ice Age, preferably a new edition of that most interesting and stimulating classic by Dr. W. B. Wright, "The Quaternary Ice Age."

To mention all the investigators whose work has enabled the above summary to be made would manifestly have been impossible. The facts used here as my premises have been provided by my geological confrères; if the conclusions drawn from them are inadmissible the fault is mine.

(*b*) Miss M. E. J. Chandler—*The Effect of the Southern Extension of Glaciers and Ice-sheets on the Pre-Glacial Vegetation. The Nature of the Flora as revealed by Plant remains associated with Glacial and Inter-Glacial Deposits.*

By a study of the living flora of Britain and of its relationship to other floras, much may be inferred of its history and origin, but the only final and conclusive evidence, the incontrovertible proof of all theories, must rest with the plants themselves as still represented in the fossil state.

Our knowledge of the British flora during the Pleistocene is incomplete and fragmentary, for it is based on relatively few floras, many of which are small ones. Each such flora is but a chance sample of the vegetation of a particular restricted area during a particular limited time. No continuous sequence is known either in space or time, the floras available being derived from scattered isolated localities, representing, as it were, lucky dips extracted from a past largely hidden by the bran of ignorance.

Four striking features do, however, stand out in any general consideration of Pleistocene floras.

(1) Many contain elements either no longer native in these islands or to-day very markedly restricted in geographical or altitudinal range.

(2) The floras when compared with one another are by no means uniform in general character. It can be shown that the differences are not merely due to geographical differences of latitude and altitude such as might be expected if one sample were from the high Pennines or the mountains of Perth, and another were from South Devon or Cornwall.

(3) A given flora may differ in general character from the living flora now growing in the same locality.

(4) Floras from similar localities which indicate differences of climate are found to be of different age, although floras which indicate similar climatic conditions need not necessarily be of the same age.

There is no such thing as *a* Pleistocene flora of Britain, for the flora was ever changing, successive floras varying in composition and relationship with changing conditions; owing their character to climatic conditions inducing migration or causing extermination, geographical conditions facilitating or hindering such migration, and biological characteristics such as may affect capacity for migration.

We have heard from Mrs. Reid that as the result of gradual cooling of climate and building of new barriers in the later Tertiaries, those elements in the older British flora characteristic of the East and of North America had been, in great measure, eliminated. In the Pre-Glacial Cromer Forest bed are found the remains of a vegetation closely akin to the living lowland

flora of Britain as represented about the Norfolk Broads to-day, but differing in this important respect: together with the common British plants a few more southern species are associated, *e.g.*, an extinct species of *Corema* allied to that now occurring on the coasts of Spain and Portugal and in the Azores, *Hypecoum procumbens*, now found in South France and the Mediterranean region. Other plants no longer native, but occurring in the Cromer Forest flora, are *Ranunculus nemorosus*, *Trapa natans*, *Naias minor*, *Picea excelsa.*

From evidence other than botanical we learn that subsequent to the deposition of the Cromer Forest bed cold conditions exercised a marked influence in Britain, and there is reason to believe, as we have heard from Professor Boswell, that cold conditions recurred on several occasions. An almost inevitable inference is that the cold of these glacial episodes eliminated many elements of the Pre-Glacial flora, although some of the more hardy may have persisted in the non-glaciated southern parts. But whenever conditions once more became favourable the land appears to have been re-colonized. Evidence of re-colonization is represented by the dry-soil floras of West Wittering and Clacton attributed to the Inter-Glacial period which succeeded the cold associated with the formation of the Great Chalky Boulder Clay. These floras in general character resemble the living lowland flora of Britain. They also resemble the Pre-Glacial Cromer Forest flora, although they probably point to drier conditions either of climate or habitat. Thus Clacton contains 41% of dry-soil species, Cromer only 31%. The exotics of Cromer have largely disappeared, but *Naias minor* and *Picea excelsa* occur. Nevertheless, so far as our limited knowledge of Inter-Glacial floras goes, some plants either never returned, or if they did so, they must again have been eliminated, for they no longer live in Britain. The absence of these plants at the present time is not in all cases due to unfavourable conditions of climate; possibly they have been out-raced by hardier and abler colonists which repossessed themselves of the lost territory with greater ease and rapidity.

Apart from inference as to the effects on the flora of those glacial episodes, during which the land was, to some extent at least, ice-covered, we have in local floras themselves definite proof that the glaciation of Britain did profoundly alter the character of the vegetation. In illustration of this statement I refer to four Pleistocene floras, two which are very small and not definitely dated, those of Bovey Tracey, Devon, and Bembridge, Isle of Wight, and two which are larger and referred to the late Pleistocene, those of Barnwell and the Lea Valley. Their true significance can only be recognized if they be compared with the present-day native floras of these respective lowland localities, although they

certainly do not indicate the maximum effect of glaciation on the British flora. At Bovey Tracey, Heer and, later, Nathorst, recognized the cold-loving dwarf birch and bearberry in abundance. At Bembridge Mrs. Reid and I determined a small flora in which the predominant element was *Ranunculus hyperboreus*, an essentially arctic and sub-arctic species. It was associated with spruce, birch, and raspberry. In the more extensive floras of Barnwell and the Lea Valley a cold-loving type of vegetation is also revealed. In such floras individual determinations may be discredited by further work, the exact degree of cold indicated is an open question, but when due allowance for such considerations has been made there remains the fact that these floras, taken as a whole, are markedly different from those of the same localities to-day, where typical lowland plants now occur, and are markedly different from the temperate Pre-Glacial and Inter-Glacial floras to which reference has already been made, in that they include northern or arctic forms, or forms now restricted to mountain tops in these latitudes: such plants as *Papaver alpinum*, *Dryas octopetala*, *Betula nana*, *Carex capitata*, *Saxifraga oppositifolia*, *Ranunculus aconitifolius*, *Arctostaphylos*, *Oxyria*, etc.

No complete survey of the imperfectly known Pleistocene plant beds of Britain has been attempted, but the few examples discussed serve to illustrate my statement that there is no such thing as *a* Pleistocene flora of Britain. The flora has changed in response to changing conditions so that during the Pleistocene there has been a succession of different floras. We must therefore conclude that the British flora of to-day is no stable unit, for from experience of the past we know that any change of conditions in the future is again bound to modify its characters.

IV—Post-Glacial Floras

(*a*) Dr. H. Godwin—*Vegetation Phases Reconstructed from Pollen-Analysis of Peat.*

The question of the origin of the British flora is essentially an historical one, and possibly the most direct way of approaching it lies in the investigation of Inter-Glacial and Post-Glacial deposits and their content of identifiable plant remains. A most fruitful method of pursuing such inquiries into the vegetational history of the recent past has been discovered in the technique of pollen analysis. It is based on the high resistance to decay of the pollen grain membranes of many plants, notably the more important forest trees. These plants are wind-pollinated and so produce pollen in very great amounts; this is widely spread and becomes incorporated in growing deposits of all kinds. In peat bogs, particu-

larly, the pollen grains may be preserved almost perfectly and they can be recovered and recognized in preparations of peat many thousands of years old. It follows that analysis of vertical series of samples through such a deposit will yield a picture of the local changes in forest history throughout the formation of the deposit. Such analyses have in fact permitted the reconstruction in broad outline of the forest history of Europe since the last glaciation, and to some extent also through the different Inter-Glacial periods.

The story so produced is concerned essentially with trees; but these are of special importance to the present discussion not only as the British plant species whose Post-Glacial history thus becomes most fully known, but also as former vegetational dominants over much of the countryside; as such they constitute *firstly* major factors in determining the existence of other species, and *secondly* they show the most direct response to the influence of climatic changes, and serve as a means of outlining the nature of the climatic sequences which must have influenced very profoundly the immigration and distribution of the bulk of plant species in this country.

Pollen grains of the different trees may be recognized with ease, they differ from one another in size, shape, number, and shape of the germ pores, in thickness of wall, marking, and so forth.

From the results of pollen analysis applied to deposits of this country I wish to use only a small number of examples chosen to illustrate special points.

Firstly, fig. 1 shows analyses of the deposits cut through in excavations for the new graving dock for the Southern Railway at Southampton. Each type of pollen is separately expressed as a percentage of the total tree pollen. *Corylus*, the hazel, is expressed as a percentage of the total tree pollen but is not reckoned in this total. It will be seen that in the lowest samples pine and birch alone are present. Later oak and elm appear and increase in amount, and later still the alder and lime. The diminution in importance of birch, and later of pine, correspond with the increasing abundance of the mixed oak forest components and especially alder. The very large change in forest composition indicated here is recognizable not only over all parts of this country but in the greater part of the European continent. It is accompanied by a phase of extremely high hazel-pollen values which is found consistently throughout north-western Europe. There can be little doubt that the change in forest history reflects general climatic change to warmer conditions.

Secondly, the diagram may be used to illustrate the overwhelming dominance of the alder in the second phase of forest development. The

same general preponderance of alder pollen is found through so many British pollen diagrams that we can only conclude the former presence of very widespread alder woods, and this suggests in turn that large parts of our primitive woodlands were seriously waterlogged before human activities were responsible for their clearance and drainage. Such bad drainage would be a factor to be reckoned with in considering migration and survival of the British flora as a whole.

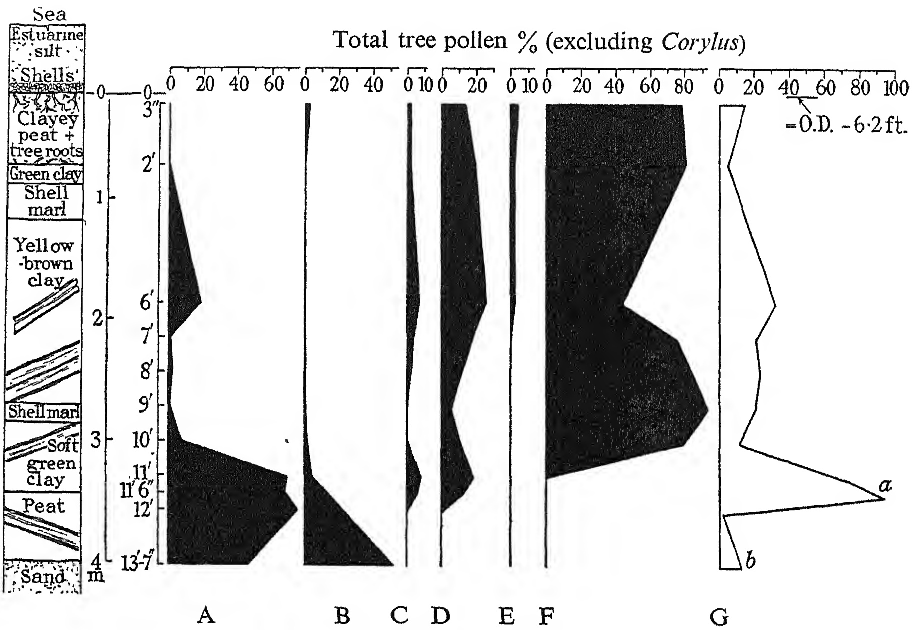


FIG. 1—Southampton S.R. graving dock, 1932. A, *Pinus* (pine); B, *Betula* (birch); C, *Ulmus* (elm); D, *Quercus* (oak); E, *Tilia* (lime); F, *Alnus* (alder); G, *Corylus* (hazel); *a*, *Myriophyllum* + *Salix* 10%; *b*, *Myriophyllum* 12%, *Salix* 10%

Thirdly, it must be noted that the basal peat here is 20 feet below present sea level, and thereby proves a Post-Glacial marine transgression of at least 30 feet since the birch-pine forest phase.

The same evidence of submergence is shown also at Swansea, in South Wales. The lower peat bed evidently comes from the end of the birch-pine phase; though oak and elm are present, alder and lime are not. This bed lies at present about 50 feet below sea level.

Of even greater interest are analyses of "moorlog" peat from the floor 120 feet below the surface of the North Sea. The samples analysed here evidently belong to the birch-pine forest phase, and indicate a period at which dry land connected the British Isles with the Continent. Exten-

sive pollen analyses of moorlog from various parts of the North Sea have thus already served to date approximately an event of the greatest importance to British plant history, namely our severance from the European mainland. The moorlog samples from the Leman and Ower banks have the peculiar interest of coming from a site from which was dredged a lump of moorlog containing a Mesolithic bone harpoon.

It has since proved possible to make other correlations between archaeology and pollen analysis, particularly in the Cambridgeshire fens where the Fenland Research Committee has been active for the past three or four years. At a site near Shippea Hill an excavation was made which yielded no less than three separate archaeological horizons stratified into the fen peats one above the other. The lowest horizon was a microlithic flint industry of late Tardenoisian type, and pollen analyses show that it occurred at the end or the birch-pine phase. Notable features of this horizon are the replacement of pine by alder, the late appearance of lime and alder as compared with oak and elm, and again the characteristic high values for hazel pollen. Well within the oak-alder forest period was found the Early Neolithic horizon.

At the same site but in an upper peat bed occurred also an archaeological horizon of the Early Bronze Age. The pollen analyses show that this, too, lies in the phase of dominant oak-alder pollen, but above this horizon we may note substantial amounts of *beech* pollen, a tree now encountered for the first time. It will be of interest to recall this later.

Pollen analyses have also been made through a section of a peat bed which lies below a " roddon," the extinct channel of a river which was flowing in Romano-British times. This pre-Roman peat shows some very characteristic features, notably a secondary pine-pollen maximum, closely followed by higher birch-pollen values, and associated with abundant ericaceous pollen. Definite though small amounts of *beech* pollen are also present.

It seems highly probable that the buried pine forests of the Cambridgeshire fens are all referable to this period. This probably also corresponds with the pine forest horizons in Scottish and the North of England mosses, with the Bronze Age pine forests described by Professor Jessen from the Irish bogs, and with the shore peats of high pine-pollen content on the Lincolnshire coast, which were found by Professor Swinnerton to contain a Halstatt (late Bronze Age) industry.

Unfortunately it has not yet proved possible to determine whether this pine is variety *Scotica*—the native race of Scots pine in Scotland—or whether it may possibly be related to the subspontaneous pine now growing on East Anglian heaths.

Fig. 2 shows analyses from the peats of the Little Ouse Valley, on the eastern border of the fens, and it illustrates in the one figure both the early birch-pine phase and the later, and smaller, pine-birch phase mentioned above. If this later pine-birch phase is indeed a constant horizon in the fens, and it appears to be so, it will be a very important feature in our Post-Glacial forest history. Not only may it have important climatic implications, but when more closely dated it will serve as a very valuable chronological index. Already it appears to indicate one fact of considerable interest—as can be seen from the figure, beech pollen appears

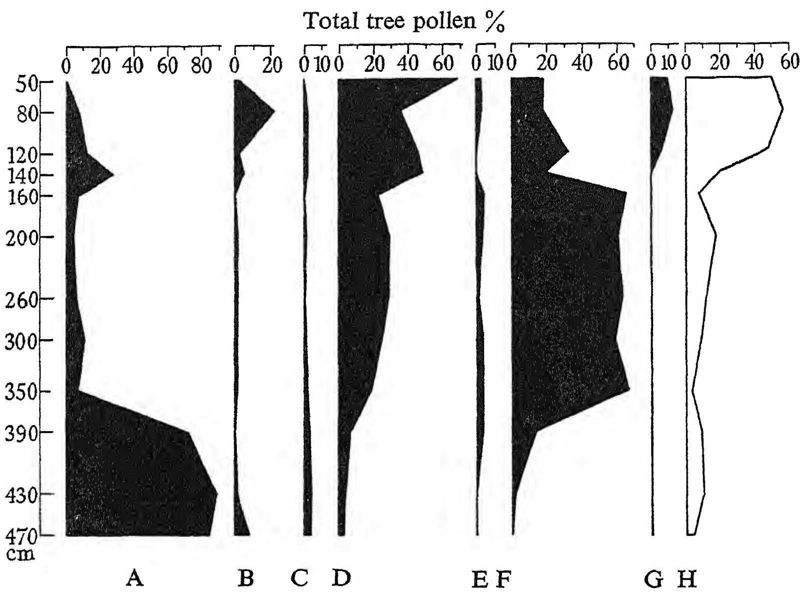


FIG. 2—Wilton Bridge, Little Ouse, 1935. A, *Pinus* (pine); B, *Betula* (birch); C, *Ulmus* (elm); D, *Quercus* (oak); E, *Tilia* (lime); F, *Alnus* (alder); G, *Fagus* (beech); H, *Corylus* (hazel)

in substantial amounts at about this time. It has been said that this tree is a Roman or post-Roman introduction into Britain, but the pine maximum has been shown to be pre-Roman, and these data would appear therefore to establish the pre-Roman Age of the beech also.

The same features are shown in the analyses of Wicken Sedge fen. Here again is the secondary pine-birch maximum, and closely associated with it is the beginning of substantial amounts of beech and hornbeam pollen. As a preliminary estimate it may be said that beech and hornbeam appear to have extended in this part of Britain at about the middle or end of the Bronze Age, a dating which agrees well with the known extension of beech and hornbeam woods across North-Western Europe.

A diagram from Methwold fen lends confirmation to this view. It shows the horizon at which a late Middle Bronze Age spear-head was found, and this level corresponds with the appearance of small amounts of beech pollen. The base shows the early birch-pine phase again—the so-called Boreal period.

Before leaving these diagrams I may draw attention to a last point of interest, namely the prevalence of lime pollen throughout the later Post-Glacial period. This is of interest in view of three facts: (1) the present restricted distribution of natural *Tilia* in Britain; (2) the statements that seedling establishment by *Tilia* is exceedingly rare in this country; (3) the remarkable fact that of the hundreds of *Tilia* pollen grains examined by my wife and myself in East Anglian peats, all have been *Tilia cordata*. If *Tilia platyphyllos* is indeed native in the west of England as it is said to be, it can scarcely have migrated there in Post-Glacial times by a route near the East Anglian fens. It would be of very great interest to trace the history of *Tilia platyphyllos* in the peats of South-West England to see how long it has been present there.

Time has forced me to confine my remarks to the Post-Glacial deposits, to pollen, and to trees, but other sub-fossil plant remains in bogs of all ages are of the most direct importance in discussion of the present problem. I need only recall by way of example that Jessen and Milthers identified, in peat beds of the last Inter-Glacial period in Denmark, remains of *Brasenia purpurea*, *Aldrovanda vesiculosa*, and *Dulichicium spathaceum*, the first and last of these plants are now only found outside Europe altogether, and *Aldrovanda* is a central European plant. In this country spruce pollen has been found in Inter-Glacial beds at Hoxne and Kirmington in Lincolnshire. Thus peat deposits may supply not only evidence of migration and climatic change since the last Ice Age but also of extermination during that period. I believe that the examination of bog stratigraphy and the plant content of bogs will very amply repay intensive development, not least in relation to such problems as the origin of the British flora.

(*b*) *Sources from which the Present Flora was Derived*

(i) Mr. A. J. Wilmott—*Evidence in Favour of Survival of the British Flora in Glacial Times.*

Time necessitates that I must restrict myself to a few crucial facts and merely illustrate the types of evidence.

One great trouble in this problem has been that interpretation of fact so far outweighs the actual evidence. For example: the evidence is—

Betula nana found at Bovey Tracey in South Devon, and with *Salix herbacea* and other northern species in the Cam and Lea valleys. One common interpretation is—Arctic Tundra in the south of England, British flora destroyed. But why not equally well *Betula nana*—living on Dartmoor, remains washed down to Bovey Tracey—and living on the Saffron Walden Hills, remains washed north and south to Cam and Lea? If we examine the contents of these so-called arctic beds we find that the term "arctic" is such an exaggeration that it is difficult to condone the persistent use of the word.

The arctic flora does not consist of all species which can be found inside the Arctic Circle. This circle is drawn at $67\frac{1}{2}°$ on a geometrical basis which bears no real relation to the flora properly called arctic. The true arctic flora is a fairly definite assemblage of species which inhabit a region limited by a line drawn a little beyond the tree limit. These species are only occasionally found south of the Arctic Circle, but a large number of "boreal" species occur north of that circle. In Lapland, with a warm summer, there is a rich flora well inside the Arctic Circle. If the term "arctic" is used to cover all these boreal species it becomes meaningless for our discussion, for it is obvious that the presence of such boreal species affords no evidence whatever of the extinction of the flora.

If we examine the list of species found in the so-called arctic beds of the Lea Valley, we find that the majority of the species are such as grow in the same area to-day. Most of the remainder occur now in Perthshire. The heading of the first column in the Barrowell Green list—"plants reaching Arctic regions"—is of the nature of a leading question, for it supplies the answer hoped for. And in doing so it invites misjudgment to such an extent that it has been stated (even recently by Professor Matthews) that the majority of the species in these beds are arctic species, which is simply untrue. To allow of a proper judgment being given the real distributions of the plants should be given, for some of them reach the Mediterranean. It is more important to observe that the list includes *Potamogeton densus*, which to-day reaches its northern British limit in the southern part of the Scotch lowlands; this indicates fairly definitely that the climate of the time when the "arctic" beds were deposited was not more severe than that of the Scotch lowlands to-day. The Hoxne list so often mentioned confirms this point of view, though it is a little colder and possibly belongs to the colder dry Loess period.

The record of *Ranunculus hyperboreus* at Bembridge in the Isle of Wight indicates that the deposit was formed in a bog kept cold in summer by melting snow. It does not give the indications of general climate attributed to it in spite of the associated species. And again I do not

feel that the evidence has been examined with an open mind. The authors state: "We were anxious to obtain confirmatory evidence of climate, if possible, but failed to do so." Why not accept the evidence of the forest trees found in the same bed? *Ranunculus hyperboreus* occurs in Finland to 64° north, where the flora is mainly composed of the species most common in the British Isles.

Table I is an analysis of the flora of Finland in relation to that of Britain. The large majority of species of phanerogams occur in Britain to-day, and the great majority of these are the common widely spread species of which the mass of our flora is composed. They are buried under snow for a long period in the winter, but they are *not* exterminated. They are just those species which might well survive in southern England during the glaciation.

The climate in southern England was warm enough to bring the glaciers to a standstill, and the evidence of the sub-glacial streams and large outwashes of gravel indicate summer warmth. Temperatures of 60° below zero have been mentioned, merely because of a figure obtained in Spitsbergen, a peculiar comparison to make in view of the enormous difference in latitude. Considering its geographical position, England may well be presumed to have had then, as now, a *comparatively* oceanic Atlantic climate, in which Atlantic species might survive even if they could not do so to-day in the continental climate of Finland. I would point out that the associate of *Betula nana* at Bovey Tracey, which was identified as *Salix cinerea*, is in fact the common species which till recently has in this country been known by that name. But it is not *Salix cinerea*; it is a wide-ranging *Atlantic* species, *Salix atrocinerea*, originally described from Portugal.

There seems therefore no reason whatever for accepting the assumed arctic climates and temperatures. The contents of these so-called "arctic beds" are, on the contrary, definite evidence of the survival of those species found in them, some of which will not now grow north of the Scotch lowlands. That seems the obvious interpretation of the evidence, and it is for the opponents of that view to provide *evidence* if a less obvious interpretation is to be accepted.

The pollen analyses of peat detailed by Erdtman provide no evidence contrary to my interpretation. In the most interesting area from the point of view of our discussion—the south of England—very little information has been collected, but there seems to be no evidence even of the extermination of the forest. I am told that I am misinterpreting this evidence: that these records do not refer to the Glacial period itself. My only object in citing these data is to point out that they provide no

TABLE I—FLORA OF FINLAND COMPARED WITH THAT OF BRITAIN

	Br.	nB.	nB * (Ar.)
Coniferae	3	2	—
Typhaceae	2	—	—
Sparganiaceae	4	—	—
Potamogetonaceae	20	1	—
Alismataceae, etc.	9	1	—
Gramineae	45	12	12
Cyperaceae	65	22	7
Spathiflorae	5	1	—
Juncaceae	17	3	—
Liliaceae	11	2	—
Iridaceae	1	—	—
Orchidaceae	21	5	—
....			
Salicaceae	13	7	1
Myricaceae	1	—	—
Fagales (and *Betula*)	6	2	—
Ulmaceae	1	1	—
Urticaceae	3	—	—
Polygonaceae	18	1	1
Chenopodiaceae	11	2	—
Montia	1	—	—
Caryophyllaceae	41	9	5
Nymphaeaceae	3	—	—
Ceratophyllaceae	1	—	—
Ranunculaceae	22	10	6
Papaveraceae and *Fumariaceae*	1	3	1
Cruciferae	37	8	4
Droseraceae	3	—	—
Crassulaceae	4	1	—
Saxifragaceae	14	1	—
Rosaceae	32	10	—
Leguminosae	28	7	3
Geraniaceae and *Oxalidaceae*	8	2	—
Linaceae	1	—	—
Polygalaceae	2	—	—
Euphorbiaceae	4	1	—
Callitrichaceae	3	—	—
Empetraceae	1	—	—
Aceraceae	—	1	—
Balsaminaceae	1	—	—
	463	115	40

	Br.	nB.	nB. (Ar.)
Rhamnaceae	2	—	—
Malvaceae	1	—	—
Tiliaceae	1	—	—
Hypericaceae	3	—	—
Elatinaceae	1	2	—
Cistaceae	1	—	—
Violaceae	5	5	1
Thymeliaceae	1	—	—
Elaeagnaceae	1	—	—
Lythraceae	2	—	—
Onagraceae	6	1	1
Halorrhagaceae	3	—	—
Hippuridaceae	1	—	—
Umbelliferae	18	3	3
Cornaceae	2	—	—
....			
Ericaceae and *Pirolaceae*	16	4	3
Primulaceae	8	3	—
Plumbaginaceae	1	—	1
Oleaceae	1	—	—
Gentianaceae	7	—	3
Convolvulaceae	2	—	—
Polemoniaceae	1	1	—
Boraginaceae	13	4	1
Labiatae	23	3	—
Solanaceae	4	—	—
Scrophulariaceae	30	3	5
Lentibulariaceae	5	—	1
Plantaginaceae	5	—	—
Rubiaceae	5	—	—
Caprifoliaceae	3	1	—
Adoxa	1	—	—
Valerianaceae	2	1	—
Dipsacaceae	2	—	—
Cucurbitaceae	1	—	—
Campanulaceae	8	1	1
Compositae	71	15	10
Diapensia	—	1	—
Vincetoxicum	—	1	—
	260	51	30

723 are British species, and include the mass of all the common plants.
236 are not British; of these 70 are Arctic, leaving 166 mainly more eastern and boreal species.
——
959

* Br., British; nB., not British; Ar., Arctic.

evidence whatever of arctic climate and extinction—not even in Argyll. I say definitely that the statements made by Erdtman concerning climate in Britain are quite at variance with the facts which he details; the data do not warrant any mention of arctic climate, and raise no obstacle to the assumption of survival.

Another line of evidence is from the mode of occurrence of local species. These do not behave like immigrants, which normally spread (*cf. Elodea* and *Matricaria suaveolens*). They behave like relicts, and are generally accepted as such elsewhere in Europe. To obtain *Bupleurum opacum* one must visit precisely the same spots near Torquay or on Berry Head as were visited for them a century ago. *Koeleria vallesiana* was found by Dr. Druce in the Dillenian herbarium with locality Brean Down near Weston-super-Mare. It was unknown as a British species. Searched for, it was found immediately, but is not known elsewhere. These relict species do not spread.

With *Bupleurum opacum* at Torquay grows *Helianthemum polifolium.* This grows also with the *Koeleria* near Weston accompanied by *Trinia vulgaris*, which grows with the *Bupleurum* on Berry Head. These are all rare species with no or few other British stations, and indicate the way in which these relicts occur. With one is found another—not the same in each case, but all of the same distribution type.

What is there about these localities that peculiar plants should be found there? It is only necessary to recollect the reason why such names as Torquay, Bournemouth (where till recently *Simethis bicolor* occurred), and Weston-super-Mare are familiar to obtain what appears to me to be the *prima facie* answer. They are specially clement spots, and may well have been so in Glacial times also. Certainly the species concerned have no relation to each other except whatever historic accident is responsible for their association. The occurrence of *single* species might be explicable as due to ecological causes, but the association of unrelated species occurring like relicts cannot be so explained away. They must, in my judgment, be given an historical explanation.

In many parts of Europe alpine plants occur (chiefly on limestones—which afford the drainage they need in winter as well as summer warmth) at low elevations alongside thermophilous plants. This association is generally supposed to be a relic of the Glacial period, the alpine species having come down at that time and persisted, and being therefore termed de-alpines. In the west of Ireland we find *Neotinea intacta*, a Mediterranean species, growing up through *Dryas*, one of the truly arctic species in the sense of being a normal constituent of the Arctic flora. But if we examine the map made by Professor Matthews to show the " arctic bed "

occurrence of *Dryas* and the present-day occurrences, we see that the extent to which the species was then found further south roughly agrees with the evidence from the contents of the beds, viz., rather less than the distance from the Lea Valley to Perthshire.

Another type of area in which very local species are found is the "unglaciated" area. Professor Fernald has shown this to occur in North America, and forced his at first incredulous and antagonistic geological colleagues to accept his view that these plants are where they are because they survived the glaciation on the unglaciated areas.

Our own flat-topped unglaciated mountains bear similar relics. On Ben Bulben, besides the arctic *Arenaria ciliata*, peculiar forms of *Polygala vulgaris* and *Thalictrum minus* occur. Ingleborough bears *Dryopteris Villarsii*, and is the locality given for two unconfirmed records. These may prove to be errors, but they may, as in other cases, yet be found. The Cross Fell area bears *Myosotis brevifolia* (endemic).

The most remarkable assemblage is found in unglaciated Upper Teesdale. Several species have here their only known station in the British Isles, or in Britain, or in England. Some are boreal species, but not all. Their only relationship is that they all occur here on this unglaciated area. I offer the following axiom: *Any fact of distribution which can be related to topographic fact of one period only of earth history must be regarded as a product of that period.* It seems incredible that these species could have migrated over intervening areas from their nearest

TABLE II

Viola rupestris	only British localities.
Alsine stricta	
Juncus alpinus	
Cystopteris alpina	
Gentiana verna	only also in W. Ireland.
Potentilla fruticosa	
Myosotis alpestris	only English locality.
Senecio spathulaefolius	southern species.
Helianthemum canum	
Dryas octopetala	
Polygala amarella	
Thlaspi silvestre	
Saxifraga Hirculus	
(*Alchemilla pastoralis*)	

stations, have settled on unglaciated Teesdale, and have died out over the routes travelled. They are there because they survived glaciation there, like the similar examples in North America. The suggestion that the peculiar Teesdale plants can be explained by the occurrence of peculiar ecological conditions or by the occurrence of the peculiar " sugar limestone " is not borne out by the habitats of these plants. The *Viola rupestris* grows on the sugar limestone there, and the *Alsine stricta* is in a stream-source marsh near by, but the *Juncus alpinus* is on riverside rocks, the *Gentiana verna* in the lower grassy areas, etc., the *Potentilla fruticosa* on the river gravels and banks of the Tees, and the others have their own distinct habitats, some of them miles apart. It is impossible that Teesdale should produce a *series* of unique and peculiar ecological habitats.

If these plants did survive the glaciation in Teesdale, they indicate the climate of the time : not arctic. We must assume tremendous precipitation in winter, of snow, but reasonable summer warmth, *i.e.*, the kind of climate that might be postulated on a basis of Dr. Simpson's suggested explanation of the glacial period.

There is no need for me to discuss the so-called " southern element " in the British flora, or that part of it known as " Lusitanian." The pros and cons are well known; the question is whether they survived or immigrated.

I would make only the following points:—

(1) we do not know for certain the land limits in southern Ireland in glacial times;

(2) the Kerry glaciation was only local glaciation in an area of great precipitation;

(3) most of the Lusitanian species will grow at considerable elevations —*Saxifraga umbrosa* to the top of Brandon mountain;

(4) *Saxifraga umbrosa* and *S. geum*, both from Ireland, in the open in my London garden, survived the snowy winter of 1927-28 and the hard, dry winter of 1928-29: but *Saxifraga umbrosa*, from the Pyrenees, was killed;

(5) Dr. Praeger has extended previous examination of these and other Irish species as regards the possibility of their seeds being wind borne or water borne. The results of these experiments are antagonistic to the supposition of the immigration of these species by these means, *i.e.*, since glacial times. Those who best know their Irish fauna and flora in the field believe that this element survived the glaciation.

The last line of evidence I will mention is the existence of endemic species. Whence could these have immigrated? They are of relict types, *i.e.*, they are local, and often have fairly close allies occurring similarly elsewhere, giving indication that they are survivals from an older

more widely spread stock. Thus there are the subglabrous allies of the common *Arabis hirsuta*, one (*Arabis Brownii*) confined to a few spots in western Ireland, another confined to the Baltic area (I think the distribution will prove to follow the line of the old Baltic river), a third in the Alps. This must surely be a survival.

Fumaria occidentalis, confined to Cornwall, belongs to a series of Mediterranean Fumitories, the commonest member of which is *F. agraria*. How can such a plant be an immigrant? And if this species survived, the others could have survived.

To me it seems much easier to see possibilities of survival, in spite of the record of *Ranunculus hyperboreus* at Bembridge, than, on a hypothesis of extinction, to offer any explanation of the affinities and occurrence of this endemic Fumitory in Cornwall.

I therefore ask:—

Are the common British species exterminated in Finland because *Ranunculus hyperboreus* occurs?

Is the flora of Finland exterminated because it is buried under snow in winter?

Is the flora exterminated because it adjoins the foot of a Swiss glacier or that of the Malaspina glacier in Alsaka?

Is the flora of Braemar exterminated because it is associated with *Betula nana* and *Salix herbacea* on the surrounding hills?

Therefore, in conclusion, does the *evidence* indicate the extermination of the British flora in Glacial times? If we leave the plants themselves to answer, I believe we shall find that they answer definitely No!

(ii) E. J. SALISBURY, F.R.S.—*Are most of the present British Plants Post-Glacial Immigrants from extra-British Regions with possibly some Human Introductions?*

The problem as to what proportion of the present flora represents survivors of the Pre-Glacial flora is necessarily linked with the climatic conditions during the glaciations. The severity of those conditions would appear to be indicated on the biological side by (*a*) the occurrence of arctic and boreal species in Devon, the Isle of Wight, and elsewhere; (*b*) by the northward migration of alpine species such as *Gentiana verna*, *Cherleria sedoides*, etc.; (*c*) by the southward migration of temperate types.

The presence of temperate species together with boreal types in what have been termed arctic beds is no criterion of the climatic conditions during the glaciations, since these deposits were from their position obviously formed before or after the coldest periods.

The northern component in our flora, and other hardy types, may quite possibly have survived the latest if not the earliest glaciations in the unglaciated areas, but they may equally represent early Post-Glacial immigrants. It is not sufficient merely to show the possibility of such survival, and considerable uncertainty apparently exists as to the continuity of nunatak areas, but the probability of survival must also be clear before we can build any superstructure on this assumption.

One may ask can the less hardy types have become acclimatized? Since a period of four hundred years has failed to suffice for half-hardy plants, introduced into this country about the middle of the sixteenth century, to become hardy (*e.g.*, *Laurus nobilis*, *Jasminum officinale*, *Spartium junceum*, etc.), it would, I think, be rash to make any such assumption to help us out of our difficulties.

In assessing the importance of the climatic conditions the possibility that ice-free areas may nevertheless have been snow-covered for a greater part of the year must not be overlooked. We must also avoid the not uncommon error of thinking in terms of lethal and non-lethal conditions. Climatic conditions that are far removed from the lethal may, as I have shown for *Silene conica*, well-nigh inhibit reproduction. The long-continued duration of the glacial periods would have an effect that cannot be measured by that of a single winter of the same severity. Southern and Oceanic species such as *Arbutus unedo*, *Erica mediterranea*, *Ulex europaeus*, etc., are cut back or even sometimes killed in eastern and southern England during the relatively short duration of a hard winter. How much more would such species have suffered during the age-long " winters " of the glacial epochs !

The relatively steep slopes and rock faces of ice-free areas may well have provided habitats where conditions essential for assimilation could have been of a seasonal duration adequate to maintain life, but, in this connection, it must be remembered that the " compensation points " of southern types, and the temperatures necessary for flower formation, are in general higher than for northern species. Also it is quite problematical whether suitable habitats for lowland herbaceous species existed even if the climatic conditions could admit of their maintaining sufficient vigour to survive.

The association of certain uncommon species in special locations in Britain has been adduced in support of the survival hypothesis. Since, however, such " sanctuaries " are not confined to unglaciated areas it is much more probable that the reasons for such occurrence are ecological and not historic. Such a view is forced on us when we recollect that the species have remained confined to these areas during the prolonged

period that has elapsed since the area around was free from ice. Their persistent localization demands an ecological explanation and renders the relic hypothesis superfluous.

The endemics in our flora have been held by some to be relic species, but many, if not most, may be recent mutants.

The evidence at present available would appear to justify our regarding the Northern component in our flora as the most ancient, perhaps even either wholly or in part Pre-Glacial; but it would appear highly improbable that the Southern-Oceanic element or the Southern-Continental element comprise any species which survived through the glacial epoch.

The problem as to which species did actually survive may be incapable of solution, but the clue is to be sought in intensive autecological studies of the species concerned. No argument based on present geographical distribution alone can have much scientific value.

Turning to the question of immigration, the poverty of the Irish flora, consisting of about 1300 species of " vascular " plants as compared with over 2000 species in Great Britain and some 4300 in France, has been held as evidence of the inefficiency of dispersal. But a comparison of the species which are present in Ireland with those which are absent, indicates that the selection has been mainly climatic and ecological rather than biological (*cf.* Salisbury, E. J., "The East Anglian Flora," Norwich, 1932). One instance must suffice. The Southern-Continental *Vicia lutea* is absent from Ireland but the generally distributed *Vicia sativa* is present although possessing a heavier and a larger seed.

The diverse climatic fluctuations since early Post-Glacial times have each in turn probably facilitated the immigration and ecesis of a particular climatic floral element, but each must have been an adverse period so far as some other elements of the flora were concerned, thus accentuating localization to areas where edaphic conditions ameliorated the climatic complex.

If it be true, as would appear may have been so, that the oak was absent from this country in the earliest Post-Glacial times, then the fact that so heavy and large a fruited plant should have immigrated later shows that Post-Glacial conditions offered no obstacle to recolonization.

Apart from the so-called " natural agencies " of dispersal the possible conscious and particularly unconscious dispersal of seeds by man must be stressed. If we bear in mind the successive waves of human invaders upon these islands we cannot help but be struck by the fact that the colonization from western and Mediterranean Europe took place in the warm moist epoch at the close of the Atlantic period: the Bronze Age colonization from Central Europe during the sub-boreal period when the

climate was of a continental type. Thus, just at the time when the climatic conditions in Britain were most favourable to colonization by the flora of a particular part of the Continent of Europe there was a passage of human immigrants from that region. If we compare the distribution of the megalithic monuments in Western Europe (*cf.* fig. 8 of Fox's "Personality of Britain") with that of the mass distribution of the oceanic element in our flora as given by Stapf, we shall realize that megalithic man colonized the area over which these plants occur to-day, and these people may therefore quite possibly have been important agents in the dispersal of the seeds.

The phenomena of immigration and ecesis still occur, and it scarcely admits of doubt that this is part of a continuous assemblage of species which has been an uninterrupted process from glacial times to the present day. Introductions are numerous, though the proportion of survivors is probably small. The British flora is in fact a dynamic community in which even the comparatively short historic period has witnessed both extinctions and accretions, but no hypothesis as to its earlier history can be acceptable which involves directly, or by implication, the assumption of inefficiency of plant dispersal.

Dr. A. Raistrick (*communicated by Professor A. C. Seward, F.R.S.*)—The maximum glaciation of the Pennines and Lake District took place during the second and third Glacial phases (Lower and Upper Purple Boulder Clay of E. Yorks) and it is therefore important to determine the extent of unglaciated areas in the north during these periods. There is no evidence that the Lake District fells had any ice-free area, the central Great Gable and Scawfell massifs showing abundant signs of glacial action over their highest summits. On the Pennines there are four large unglaciated areas in Durham and adjoining counties, Crossfell, Killhope Law, Knoutberry Hill, and Mickle Fell. The upper limit of ice-action around these areas is marked by the disappearance of all traces of Boulder Clay and transported erratics, and of fluvio-glacial action, while the areas themselves are marked by deeper weathering of the rocks and a greater thickness of "rock-head." A total area of over 50 square miles mostly above 2000 feet O.D. is represented by these nunataks. In the Yorkshire Pennines the unglaciated areas are more numerous but less in extent between the Tees and Aire; but south of the Aire valley the main watershed of the Pennines is largely unglaciated as far south as the Derbyshire borders. Here in addition to other evidence occur many isolated pillars and stacks of unweathered grit, and a great thickness of

grit débris, the grit material being extensively kaolinized. This area is at least 400 square miles in extent, mostly above 1000 feet O.D.

Peculiar floral elements associated with the Durham unglaciated areas were described in 1931 by Dr. K. B. Blackburn.

Discussion of Dr. Godwin's Paper on Pollen Analysis of Peat

Dr. A. RAISTRICK (*communicated by Dr. H. Godwin*)—The results brought forward by Dr. Godwin from the Fen district of East Anglia are in very striking agreement with those obtained by the writer in Yorkshire, and by the writer and Dr. Blackburn in the Northern Pennines.*

At many points between the Aire Valley and the Tyne Valley (an area about 100 miles north to south and 40 miles east to west) the base of the peat deposits is found to be a highly compacted buttery peat, very rich in tree pollen, and not usually forming more than a 3- or 4-inch thick layer. This peat is marked by a very high pine-birch content (often as much as 80% pine), and an excess of hazel (to the same base, as much as 300%). This is certainly the boreal peat of the Fens, and the high hazel content suggests the upper boreal. It occurs over most of the Pennines always in small isolated areas, usually in the valley heads between 1000 feet O.D. and 1500 feet O.D., and not on the Fell summits. In three places this peat is associated with " microlithic " flint industries.

Oak, elm, and lime have been found in these peats, along with 60% of pine, but they only reach abundance along with the dominant alder of the Atlantic peats. Beech and hornbeam have been obtained from peats of the horizon upper Atlantic-sub-boreal, and late Bronze Age implements (socketed and looped celt and a socketed spear-head) from a slightly higher peat.

A second pine maximum (usually about 30%) usually occurs well below the horizon of Roman remains and in peats of sub-boreal type, again corresponding closely with East Anglia.

Glacial Survival of Plants in Scandinavia and the British Isles

G. EINAR DU RIETZ—The Scandinavian mountain flora contains, besides the ubiquitous element, a number of *centric* species occurring only in certain restricted parts of the mountain chain. These species are either northern unicentric (*e.g.*, *Pedicularis flammea*), southern unicentric

* Raistrick and Blackburn, " Late Glac. and Post-Glac. Periods in N. Pennines," 'Trans. N. Nats. Union,' pp. 16–34 and pp. 79–103 (1931, 1932); and unpublished work.

(*e.g.*, *Artemisia norvegica*), or bicentric (*e.g.*, *Rhododendron lapponicum*), and they are concentrated to certain mountain areas in a way which cannot be explained by any habitat factors, since just the same types of climate and soil are found in many other mountain areas as well. The Scandinavian distribution areas of these species are often very isolated from their extra-Scandinavian distribution. Many of them are west-arctic, distributed in North America and Greenland, often also in Iceland, rarely in Scotland, but never in the rest of Europe.

These facts have gradually caused Scandinavian geobotanists to give up the old theory of the complete glacial destruction of the Scandinavian flora and to advance the theory of glacial survival of an alpine flora in separate ice-free coast areas in Norway. This theory is supported by the common occurrence of nunatak sculpture in the coast areas indicated by the geobotanical evidence as probable glacial plant refuges and evidently only locally glaciated at least during the last glaciation. Submarine cirques and terminal moraines in arctic Norway indicate that the greater part of the ice-free coast areas lay on the present sea bottom.

The most conclusive evidence for the glacial survival of plants in Norway has been supplied by Nordhagen's recent investigations of the taxonomy and distribution of Scandinavian mountain poppies. Nordhagen has shown that the old compound species, *Papaver radicatum*, is differentiated in Scandinavia into several distinct species and subspecies with significant distribution areas, which have evidently survived the last glaciation in different ice-free coast areas from the Varanger Peninsula to South-Western Norway.

That a flora of the arctic type concerned here can grow on ice-free coast areas at the margin of a big ice-sheet is conclusively proved by the present flora living in such areas in Greenland, Spitzbergen, Novaja Zemlja, and other arctic countries. A very good example is described and illustrated by Lynge in Rhodora, 1934, from the north of Novaja Zemlja.

In the light of our present knowledge of the Scandinavian flora and glacial geology it seems not only certain that a very great part of this flora survived at least the last glaciation on ice-free coast areas in Norway, but it seems even doubtful if a single Scandinavian mountain plant was ever chased south by the big ice-sheet into Central Europe, as believed by so many early authors. The Late-Glacial relics of South Sweden are in some cases found to be nearer related to Middle European mountain species than to Scandinavian ones, *e.g.*, *Euphrasia salisburgensis*. The Late-Glacial tundra round the southern Baltic was apparently inhabited not by Scandinavian mountain plants but by Middle European ones.

The only Scandinavian mountain plant not found in the present flora of Middle Europe but reported from Late-Glacial deposits in this region, *Salix polaris*, is doubtful.

It is a well-known fact that there are many alpine plants and animals common to Scandinavia and the British Isles but entirely absent from the mountains of Middle Europe. These species speak strongly in favour of a glacial survival of alpine plants in ice-free coast areas both in Scandinavia and the British Isles. This applies also to some oceanic coast species with the same distribution type, *e.g.*, *Cetraria norvegica*. As to the more termophilous oceanic species common to Western Norway and the British Isles, a survival in Scandinavia seems less likely. But the absence of some of these species, *e.g.*, the lichen species *Pseudocyphellaria* (= *Sticta*) *crocata* and *Ps. Thouarsii*, from Europe outside the British Isles and Norway speaks strongly in favour of their glacial survival in the British Isles. The evidences for the glacial survival of the oceanic south-western flora in the British Isles have been admirably summarized by Dr. Wilmott. G. Degelius has recently dealt with the bearing of oceanic lichens on this problem, A good proof is supplied by *Sticta sinuosa*, which is found on this side of the Atlantic only in the south-west of Ireland. It is a common species of the tropical rain forest in Central and South America, and could not possibly have immigrated into Ireland in Post-Glacial time. *It is an obvious relic from the Tertiary rain forest of the British Isles.*

The theory of a glacial forest region in Western Europe south of the glaciated areas of the British Isles is supported not only by the distribution of the present flora, but is also suggested by the well-known Swedish glaciologist, Dr. Fredrik Enquist, on his unpublished map of the geographical conditions in Europe at the maximum of the last glaciation. According to Enquist, the sea-level was then at least 200 metres lower than now, which gives ample space for this glacial forest region on the present sea-bottom south of the British Isles. In Late-Glacial Fennoscandia, there was apparently only a narrow zone of tundra between the forest and the retreating ice-sheet. The famous forests in Alaska growing close to the big Malaspina glacier and even on its surface moraines already in 1889 caused Drude, and later on Brockmann-Jerosch, to assume such a glacial forest region in Western Europe. In our days birch forest grows close to the great ice-sheet in the fiords of South-Western Greenland, and evergreen rain forest with tree-ferns and *Hymenophyllaceae* grow not only close to the big New Zealand valley glaciers but also close to the really big ice-sheet in Western Patagonia. Considering all these facts together with those summarized by Dr. Wilmott, it does

not seem too bold to believe that *at least most of the oceanic flora of the British Isles survived the Pleistocene glaciations in a glacial forest region south and south-west of Ireland and England.*

Open Discussion

Dr. W. Watson—In the series of excellent examples of distribution shown by the previous speaker (Professor Du Rietz), use has been made of some lichens found in the British Isles. All the previous speakers have based their remarks on the phanerogams; no advantage has been taken of our knowledge of the distribution of bryophytes and lichens, though a great deal of this is readily available and can be easily used. Macvicar in 1910 gave some valuable information on the Atlantic and alpine plants in "The Distribution of Hepaticea in Scotland," and much additional information on the distribution of bryophytes has been collected and published since. During this discussion much additional evidence on the relationships and origin of the British flora could have been given from the distribution of bryophytes and lichens. As concrete examples the following may be mentioned.

In the "Refuge Areas" near Weston and Torquay, "in which unrelated relics occur together," there is much similarity in the bryophtes and lichens, especially in the abundance of the southern moss, *Eurhynchium circinatum*. *Helianthemum polifolium* occurs in both areas but so does *Aster Linosyris*; at Berry Head it grows near to the *Helianthemum*, and at Brean Down it grows within a foot of *Koeleria vallesiana* though over a mile away from the nearest *Helianthemum*. There are really three isolated areas near Weston, these being Brean Down, Purn Hill, and Uphill. On Brean Down and Purn Hill *Helianthemum*, *Koeleria*, and *Eurhynchium circinatum* occur together, but the *Aster* is absent.

The most southern station for *Saxifraga oppositifolia* is Craig Cerig Gleisiad in Brecon, and this is the only place south of Perthshire where the hepatic *Lophozia heterocolpa* grows.

At Lakenheath in Suffolk the occurrence of *Lophozia Hatcheri* and, in a lesser manner, the presence of *L. barbata*, *Stereocaulon evolutum*, and *Cladina rangiferina* supplement the evidence of the Glacial Drift. *L. Hatcheri* is frequent in the Highlands of Scotland, rarely occurs in Wales and North England, and its occurrence at Lakenheath can only be as a relic. The *Cladina* mentioned is the true reindeer moss, the records of which from southern England are very doubtful, being based on forms of the common *C. sylvatica*. The *Stereocaulon* has not been found east

of a straight line drawn from Devon to Yorkshire. Then the presence of *Gentiana verna* in Teesdale can be correlated with the recent discovery of *Moerckia Blytii* in that district. This is an arctic-alpine liverwort unknown elsewhere south of Perthshire. The isolation of *Lophozia lycopodioides* at Ingleborough and of some lichens (*Gyrophora*) on Shap granite in Westmoreland is also significant.

Dr. G. C. Simpson—Meteorology alone could throw little light on past climates; it needed the geologist, the palaeontographer, and the pre-historian to say what the actual conditions were, and then the meteorologist might be able to explain and co-ordinate the observations.

Mrs. Reid had said that in eocene times the climate of the British Isles was almost Malayan in character. That would mean an appreciable increase in temperature above that existing to-day. It is almost impossible to conceive such an increase of temperature as due to any geographical change; for at present the British Isles is situated in one of the most extreme positive temperature anomalies which exists anywhere, owing to its peculiar geographical position, and it is almost impossible to conceive any change in sea and land or in ocean currents which would increase that anomaly. The increase must therefore have been due to a change in solar radiation, and if that is so the work he had done on solar and terrestrial radiation would lead him to conclude that the increase of temperature must have been accompanied by heavily overcast skies and much precipitation. This would lead to a great reduction in daily and annual variation in temperature. Was that conclusion supported by the geological evidence? He understood why Professor Boswell had confined himself to the evidence of glaciation in the British Isles; but the problems of past climates cannot be solved by evidence from one small locality. During the Pleistocene Period we know that there were a succession of glaciations in the British Isles, the Alps, and Northern Europe, to say nothing of other parts of the world. Now those glaciations cannot be unrelated; the meteorologist cannot conceive of a glacial history in the British Isles independent of the glacial history of the Continent. There must be correlation between the various glaciations, and in his opinion the greatest problem in the study of the ice ages is to correlate at least one of the glacial epochs in the British Isles with one of the glacial epochs in the Alps and one of the advances of the Scandinavian ice-sheet. He trusted that Professor Boswell would be able to give them one such correlation. Professor Boswell had spoken of the great outwash of gravels due to the melting of the ice-sheets. He wished to point out that the outwash from an ice-sheet would be little more when the ice-sheet was retreating than

when it was at its maximum or even when it was advancing. The outwash was a seasonal effect and would always be great in the summer on the margin of a vast ice-sheet ending on land; while the retreat of the ice-sheet was a small differential action spread over thousands of years and therefore the summer flow of water would not be appreciably greater as the result of the retreat.

With regard to Dr. Godwin's contribution, he wished only to remark that the changes of climate which Dr. Godwin dealt with were of a different order of magnitude from those which produced the Ice Age. The rapid change from Arctic conditions to the warm, wet Atlantic period could be explained as the consequence of the removal of the secondary effect of the ice-sheet when, if the theory he had put forward was correct, the solar energy was greater than now and the climate, when freed from the effect of the ice, warmer and wetter than at present.

From the Atlantic maximum the climate as a whole has become colder and wetter owing to a decrease in solar radiation. On this smaller changes, probably due to geographical change, were superimposed. There must have been an appreciable local change of climate when the North Sea was formed, and the date of that has not yet been fixed.

Mr. Dewey—Attention was drawn to the scarcity of definite information relating to Inter-Glacial periods in England; but it was stated that in the east and south-east of the country there is evidence of a temperate climate during the cultural stages of the palaeolithic sequence, marked by Clactonian, Acheulean and early Levalloisian implements, preceded and succeeded by glacial conditions. Plant remains confirm the evidences derived from fauna, topography, and sequences of deposits, but fail to indicate the existence of other Inter-Glacial periods in a satisfactory manner. Comparison with Danish evidence shows the poverty of British deposits. It is held by Danish geologists that three glaciations and two Inter-Glacial periods are recognizable in Denmark, which they correlate with the Alpine Mindel, Riss, and Würm glaciations and inter-glaciations. In lake deposits lying between glacial beds well-defined sequences of fossil plant assemblages have been found. Those of the Mindel-Riss interglaciation correspond flora by flora with those of the Riss-Würm, and show the gradual replacement of the arctic by the cold-temperate and that by warmer-temperate plants, and then the reversal of climatic conditions, the *Betula nana* and the *Salix phylicifolia* assemblage being replaced by the *Betula pubescens* and the *Pinus* group and these in turn by deciduous trees characterized by the oak. A similar sequence of floras was repeated three times with but apparently little change. These facts are difficult

to reconcile with the view that a well-established flora cannot easily be destroyed or driven out and return again without modification or serious loss of species.

If the evidence is correct it implies a balanced coherence of plant assemblages of a marked nomadic habit. One would expect disturbance of co-ordination among such groups as they were evicted from habitat after habitat and struggled to return and reconquer lost ground again and again.

Dr. J. B. Simpson—A pollen analysis he had recently carried out of peat collected at Bridge of Earn, Perthshire, from the well-known submerged forest-bed beneath the deposits of the 25-foot or Neolithic beach agreed with an earlier one made by G. Erdtman of peat from this deposit collected at a different locality. There is a great abundance of willow pollen in the basal layers. The marine transgression occurred in early Atlantic time.

The great similarity in the tree succession in Scotland and England indicated by pollen analysis prompts the question whether we should regard this development as contemporaneous in these countries or whether the succession is similar merely because these areas experienced a similar, though not necessarily contemporaneous, series of climatic changes since the Glacial period.

In Dr. Simpson's opinion this point is important since we know that southern and even middle England were ice-free long before, perhaps several thousands of years before, Scotland. If the vegetation zones at present recognized in the peat are sensibly contemporaneous one might expect to find additional earlier zones in the southern localities for which there are no counterparts farther north.

On the other hand, if the zones have merely followed up the ice-retreat one should be chary of assuming any great degree of contemporaneity between florally similar zones in widely separated north and south localities in Britain.

K. S. Sandford—The speaker wished to associate himself with Professor Boswell's statements. A few years ago the conception of an early Pleistocene submergence of parts of this country was extremely unorthodox, but there was now a growing belief that much of East Anglia and the Thames Basin was submerged, and the West Country might be included. Much remains to be done before the nature and extent of the submergence can be proved, or the facts fully explained in some other way. For the present we cannot afford to ignore the possibility that the Pliocene flora

could have survived only on high ground. At no subsequent period, however, did ice enter the upper part of the Thames Basin. At certain times glaciers reached the Ouse-Ray watershed a little west of Buckingham, invaded the Cherwell valley as far as Banbury and the Evenlode (as Miss Tomlinson has shown) as far as Moreton. The Cotswolds were then cold uplands with mammoth fauna, the valleys clay marsh and swamp with wide gravel banks which, in warm intervals, harboured a hippopotamus fauna. There were widespread signs of frozen soil and of mud and rock flow which are reminiscent from a geological point of view of a present-day zone of tundra. In southern England highlands were therefore neither submerged nor glaciated, but there was in all probability much ground ice, though it was not proved to have been ubiquitous. How far were late Pliocene plants able to survive in such circumstances? At times a temperate flora and fauna (both molluscan and vertebrate) was established with a variable number of more northern or more southern species. Faunas appeared to be good indicators of climate, with obvious exceptions such as hardy carnivors that followed game over great distances. Some of the widely roaming mammals such as the elephants, reindeer, horse, cattle, might themselves help to disseminate plant species.

Later in the discussion the speaker endeavoured, on Professor Tansley's suggestion, to answer the question of the correlation of British and European glacial episodes. He felt that he was expressing the opinion of the majority of the geologists present when he pointed out that the sequence of a part of the Alps (*i.e.*, Gunz, Mindel, Riss, Würm) was not acceptable in other regions of the Alps themselves, and he had heard the same opinion in Poland from geologists working in the Carpathians. A fourfold glaciation could not be identified in the polar regions, nor did it seem to harmonize with the progressive glacial centres of Canada. In this country there exists a strong opinion among geologists that the successive ice-advances on east and west sides of the Pennines, in Wales, and the long-sustained glaciation of Scotland must be elucidated on their own merits. To assume for them either a single glacial epoch or a fourfold succession rigidly attached to Penck and Brückner's nomenclature, was misleading and unjustified by the evidence at present available.

Dr. R. W. Butcher—Whilst, not unnaturally, evidence for the origin of the British flora has been taken from past relics, a survey of the known changes in the flora during the last 200 years suggests that it is probable that most of our plants have arrived in Post-Glacial times.

A plant is established in two stages. First there is the continual arrival of reproductive bodies. Out of many millions only a very few

grow, still fewer reproduce themselves and even fewer establish themselves and colonize other territory from their original settlement. The quantity of plants depends almost entirely on whether the soil and climate are suitable for a particular species. Among such recent arrivals one might instance *Arenaria gothica* on Ingleborough and *Hydrilla verticillata* in the Lakes which have not yet spread far, or *Erigeron canadense*, *Elodea canadensis*, and *Impatiens glandulifera* which have colonized considerable tracts of country. If all this, and much more, has taken place in 200 years, the probabilities of profound changes in the British flora in the 20,000 years since the Ice Age—changes that could wipe out the glacial survivals—can be easily realized.

Similarly with endemics. Why should they be considered to have arisen only in Pre-Glacial or Inter-Glacial times ? I might suggest that *Spartina Townsendi* is, or was, a recent endemic, since it occurs naturally only on the sea-coast on either side of the English Channel. There is positive evidence that this plant originated from hybridization in the neighbourhood of Southampton Water from two other species, one recently introduced.

If this has happened within the last 100 years, why should not other endemics have arisen in the comparatively longer period since the Ice Age ?

Dr. W. B. Wright drew attention to the obvious difference in the employment of the term " Newer Drift " in England and on the Continent. The Newer Drift on the Continent is limited by the Brandenburg Moraine and characterized by much greater youthfulness of topography, and in particular by the occurrence of open unsilted lakes. There are no unsilted lakes of glacial origin near the periphery of the Newer Drift as we undertsand it in England. They are confined to areas much nearer the centres of dispersal, the Southern Highlands, the Southern Uplands, and the English Lake District, and as yet no attempt has been made in this country to separate the later glaciation thus indicated. In Ireland, however, Mr. Farrington has succeeded in proving that the local glaciers of the Wicklow Range were definitely later than the Newer Drift as traced by Professor Charlesworth, and recent excavations of the famous Ballybetagh locality on the latter drift show a climatic oscillation corresponding to Mr. Farrington's later glaciation. As this last glaciation on the Continent is considered to be separated from that which preceded it by a well-marked Inter-Glacial period, it seems clear that in dealing with the history of the British flora we have to consider the effect on it of a glacial period in which the greater part of Great Britain was free from ice.

Another factor which must have had considerable influence was the phase of dry cold which apparently accompanied the oncoming of each glacial period. There are several lines of evidence which point to unique climatic conditions during the growth of the ice-sheets. Our hill slopes show no systems of glacial drainage channels which were formed during advance, and, at the maximum extent of the ice, glacial drainage was feeble or non-existent. Petrological analyses such as we owe to Boswell, Solomon, and Raistrick have demonstrated that the sand and gravels interbedded with the drift sheets of the East of England are associated with the Boulder Clay below rather than with that above them, or in other words are products of retreat not of advance. Again, on the Continent, where loess formation was possible, it was the concomitant of oncoming and maximum glaciation and not of retreat. The fauna of the loess was that of cold steppe in which the jerboa and the lemming, the musk-ox and the saiga antelope could exist together. No such association exists at the present day except perhaps in a modified form along meridionally directed mountain ranges which extend through the taiga from tundra to steppe. Nehring considered the association of tundra and steppe forms so difficult that he had recourse to migration to explain it; but recent researches have shown it to be widespread and intimate. The existence of cold steppe over protracted periods during the Ice Age must have profoundly affected the flora of Europe in a direction which is difficult to calculate, since we have no modern analysis to work from.

Moreover, as regards their history, the floras of Western Europe and Great Britain are one. There is no question of continuous insularity in either Glacial or Post-Glacial time.

Dr. H. Hamshaw Thomas, F.R.S.—The discussion had shown the great complexity of the problem and also the value of the co-operation of specialists on its varied aspects. The recognition of four distinct periods of glaciation by those best qualified to speak of the geological evidence was of the highest importance, especially in view of the uncertainty about Inter-Glacial periods which had existed in the past. It was hoped that it would soon be possible to establish correlations between the successive ice-sheets of Britain and those of other countries. He ventured to suggest that Dr. W. B. Wright might consider the Shropshire meres in connection with the correlation of the drifts left by the ice of the fourth glaciation.

When consideration was given to the geological evidence which had been brought forward, and to the stratification of plant remains and pollen succession in our peat deposits, it seemed clear that no fewer than eleven

changes in climate must have occurred since Pliocene times. Each change probably involved extensive modification of the vegetation and the initiation of new seres, the new conditions favouring the growth and spread of certain species at the expense of others. Some species which had successfully survived the cold of a period of glaciation in an ice-free area may have been subsequently exterminated by warmer or damper conditions. The relict species in our flora must therefore be of very varied age, and they may be expected not so much in unglaciated areas as in localities where they were sheltered from competition or otherwise favoured by the soil or topographical conditions.

More attention should be given to the effects of changes in the atmospheric precipitation, especially in view of the physical considerations presented by Dr. G. C. Simpson. When considered in the light of Dr. G. K. Fraser's recent studies of the Scottish moorlands, the much-neglected work of Lewis and of Samuelsson on the stratification of the northern peats in Great Britain gave sure evidence of changes in the precipitation and in the N/S ratio during Post-Glacial times. Probably similar evidence may be obtained from the study of the topography and the river gravels of different parts of England. These changes, which are reflected in the pollen sequences, must have affected the vegetation of the whole of the British Isles.

Little had been said as to the areas in which our genera and species had originated, but an approach to this problem was indicated by the work of some of our botanical colleagues on the Continent. From investigations of the taxonomy and distribution of the forms occurring in the Alpine region, Diels, and more recently Gams, recognize a number of different elements, which may have migrated into the region during Tertiary times from other parts, and from which the present flora was derived. Species derived from these same stocks are present in the British flora. According to this reasoning many of our plants were derived from forms which were widely distributed in the Northern Hemisphere in Tertiary times, occurring in North America and Eastern Asia as well as in Europe. Another group probably had an arctic polar distribution in Tertiary times, while a third had a Mediterranean origin. But Diels and Gams also recognize a very old oceanic element, and a considerable number of forms which probably originated in the Southern Hemisphere and may be regarded as African. In our flora this African stock is probably represented by the genera *Erica*, *Polygala*, *Daphne*, *Geranium*, *Linum*, *Helianthemum*, *Viola*, and *Alchemilla*, and by the members of the *Crassulaceae*. The time, route and conditions of their migration present interesting probems. This work, which appears to be

little known in England, gives additional evidence of the complexity of the questions under discussion.

Mr. MILES BURKITT, in replying to Dr. Simpson's plea that prehistorians should come to some conclusion in regard to the correlating of at least one well-known culture with some recognized geological level, drew attention to the recently published work at the cave of Cotencher (Neuchâtel). This site lies within the area reached by the ice of the last main glaciation of the Alps at its maximum extension. It is filled by morainic material which is definitely dated, in part from considerations of its faunal contents, to this last main glaciation. Associated with the fauna in question Mousterian tools were discovered in the morainic material. Thus the true Mousterian culture must be correlated with this glacial epoch. At the same time it should be noted that all flake-tool industries are not necessarily Mousterian in culture, and many pre-Mousterian flake-tool cultures such as the Cromerian, Clactonian, Levalloisian, etc., existed.

Attention was also drawn to the occurrence of veritable oases of gravels resting on the moving glaciers in the Karakoram region. These oases yield a definite flora and fauna, as well as showing all the phenomena of denudation, etc., yet all the time the under-topography is moving ice.

Dr. J. BURTT DAVY—Dr. Hamshaw Thomas raised a question on the origin of the African element in the British flora. Although there are three primary and very definite phyto-geographical regions in Africa, the term " African flora " is accepted by many as referring to the peculiar flora of the south-west region of the Cape Province. There is a tendency to consider that elements of this south-west Cape flora have migrated northward into the Northern Hemisphere; as this tendency appears to be growing it may be well to recall an alternative view.

There is much evidence pointing towards a definite migration of genera from the Northern to the Southern Hemisphere; the onus of proof seems to rest with those who hold that the movement has been in the opposite direction.

A few striking examples of British, and definitely North Temperate Region, genera which have migrated naturally into the Southern Hemisphere, or are traceable for at least part of the way, include *Berberis*, *Cornus*, and *Populus* which reach approximately to the Equator; and *Agrimonia*, *Buxus*, *Rubus*, *Salix*, *Sanicula*, *Thalictrum*, *Vaccinium*, *Valeriana*, and *Viola*, of each of which at least one species, sometimes more,

reaches South Africa. Some of these genera are widely distributed in the Northern Hemisphere, Europe, Asia, and North America.

Erica and *Podocarpus* are usually cited as examples of South African genera which have migrated from the Southern into the Northern Hemisphere, on the assumption that the area occupied by the largest number of species of a genus is the probable centre of distribution of that genus. There is, however, another possible explanation of these cases. If we consider that the particular species having the widest distribution of any in the genus represents the oldest living species of that genus, then we may assume that the genus had its origin somewhere within the area occupied by that species. In the case of *Erica* we have the species *E. arborea* distributed from Madeira to Tanganyika Territory by way of the North African coastal region and the mountains of Abyssinia, a range of some 5000 to 6000 miles. When we consider in addition that this same species is the largest woody representative of the genus, becoming a tree 40 feet high or more on the mountains of East Africa, there is some ground for looking upon it as perhaps one of the oldest living representatives of the genus, and we have reason to assume that the genus *Erica* probably originated somewhere within the area now occupied by *Erica arborea*. It seems reasonable to suppose that the genus migrated southward by way of the heath-lands which occupy the higher mountain peaks of the " back-bone " of Africa Here isolated species are still to be found, which have been able to do little more than survive under the dense growth of the montane rain forest, or in competition with the rank grasses of the savannah forest.

It is noticeable that as we proceed southward, the number of species of *Erica* gradually increases, as the area of temperate montane climate increases. When the south-west Cape floristic region is reached, where there are practically no turf-forming grasses to compete destructively with seedling *Ericas*, the family *Ericaceae* has run riot in the evolution of species and even of endemic genera. But these species and genera have a very limited range of distribution as compared with those of North Africa, and would appear to be of younger age.

If the genus *Erica*, or the family *Ericaceae*, had originated in the Southern Hemisphere it seems probable that such a large genus as *Erica* would occur in Australia, instead of which we there find it represented by the distinct family *Epacridaceae*. Why should not the original home of the *Ericaceae* have been in the mountains of Eastern Asia, where the family is still represented by such a large number of arborescent species ? Thence it (or an ancestor of both) might have spread southward through the Indo-Malayan region, giving rise to endemic genera from which the *Epacridaceae* may have developed in Australia. We have also much

evidence to show a definite migration between the East African and the Malayan floras, and the *Ericaceae* may have reached the mountains of Abyssinia from the Indo-Malayan region migrating thence to Western Europe and southwards to the Cape.

The genus *Podocarpus* also, though now abundantly represented in the Southern Hemisphere, may have had its origin in Eastern Asia, where some species still occur, and have migrated along the same routes.

Dr. W. B. Turrill pointed out the need for investigating the history of every species separately. Very often close taxonomic relationship does not mean close ecological relationship. Two species of the same genus may be members of quite different plant communities, and in any given part of their present distributional area they may have had very dissimilar histories. Extensive and intensive studies on *Silene cucubalus* (*S. vulgaris*) and *S. maritima* point strongly to the introduction of the former to the British Isles since the Ice Age and the survival of the latter from Pre-Glacial times. This conclusion is based not only on the present distribution and ecology of the two species but on their variations, genetical relationships, and (less convincingly) on fossil records. Detailed cyto-genetical investigations often throw much light on the history of species, and the speaker felt that much more research on the individual species of the British flora is needed before the diverse problems of its origin can be solved.

Dr. Alex S. Watt—The present or past distribution of species cannot be interpreted without taking due account of three complexes of factors, climate, soil, and vegetation, all of which change both in time and space. Among these, climate may be looked upon as the master group of factors, for it determines the direction in which both soil and vegetation develop and sets limits to that development. But between soil and vegetation (in any given stable climate) there is an intimate relation, for soil is a product of the interaction of vegetation and climate, and vegetational change brings about soil change with repercussions on the vegetation itself. At any moment in time, in a country with an immature topography, there will be, in addition to soils and vegetation units in equilibrium with the climate, other soils and plant communities moving towards the stable state. In other words, there will be immature soils and immature vegetation, and mature soils and mature vegetation, and generally speaking with time the area occupied by maturity will increase at the expense of immaturity. There is then a change in the vegetation and soils both in time and space. Add to this, climatic amelioration in time as well as the

arrival of new species, particularly dominants, and the complexity of the problem increases.

Students of vegetation are familiar with the fact that as vegetation develops the floristic *cortège* of the successive communities changes, and different species characterize different developmental stages or have a different vegetational status. In illustration I may cite the varied and relatively rich flora of the mineral soils and lochs of West Scotland, to be succeeded in time by the climax moor with its few species of higher plants; or the 90 species (many of them calcicoles) of the immature soils of Breckland to be replaced later by a dozen species or so (none calcicolous) on the mature or climax soil. Now many of the species regarded as critical in the present discussion are essentially species of immature soils (either new soils like sand dunes and shingle banks or soils maintained immature by topography and erosion) and of open or immature plant communities. Habitats suitable to some of them must at one time have been much more widespread, but with the development of the habitat, organic as well as inorganic, these species have been replaced, and broad vegetation barriers in the form of climax units, in which they are unable to gain a footing, separate suitable sites: some of these sites, although they appear suitable at the present day, may not have been so at the climatic optimum. The existence of species in their present sites need not necessarily mean continuous occupation since Inter-Glacial times, but rather that in a changing climate the soil somewhere, and perhaps at different places at different times, was open to occupation where the species were safe from expulsion by competition with other species belonging to a later stage in the plant succession. Thus present distribution alone is no guide to the past distribution of a species, but deductions from its present habitat, behaviour, and vegetational status (and this I should like to emphasize) may be applied with greater validity towards the reconstruction of a past flora.

Dr. Hollingworth felt that a word or two of caution should be given. It would be unfortunate if botanists, and others present, gained the impression that the existence of large unglaciated areas in the Northern Pennines was generally accepted by geologists familiar with the glacial deposits of the North of England. While it was impossible to go into the evidence at so late a stage in the discussion, the view that the whole of the Northern Pennines were covered by a great field of snow and ice would probably find many supporters, and much could be said in its favour. It seems rather unsound to insist on the floral assemblage of the Teesdale "sanctuary" as a relict from the last Inter-Glacial period

while the possibility of actual sanctuaries there is from a glaciological aspect a debateable question.

With regard to Dr. Simpson's request for a correlation of the English glacial sequence with that of the Alps; the difficulties appear to be largely on the Continent. There, geologists are not yet in general agreement; for instance, in recent years very diverse views have been expressed on the correlation of the North European ice-sheets with the Alpine sequence. There are also unsolved problems in the correlation of the culture stages and the glaciations. Until such questions are cleared up the use of the terms Gunz, Mindel, Riss, and Würm for British glaciations is to be deprecated.

Dr. Sandford had referred to the climatic significance of the coomb rock and allied deposits. Had those who advocated the survival of the British flora in the extreme south sufficiently appreciated the severity of the climatic conditions indicated by these widespread solifluction phenomena, which were found not only throughout Southern Britain but also beyond the Channel in Brittany?

612.647:612.2

The Croonian Lecture

Foetal Respiration

By Joseph Barcroft, F.R.S., Professor of Physiology in the University of Cambridge

(*Received May* 10—*Read May* 23, 1935)

[Plate 14]

The present lecture is an attempt to state the principal facts known about foetal respiration in the mammalia. The word " respiration " is used in a very broad sense: the transport of gases involves the circulatory system even more in the foetus than in the free organism, and therefore the principal factors in the foetal circulation must come under review.

The Placenta

The general scheme of foetal circulation is that blood deficient in oxygen goes to the placenta along the umbilical arteries; these vessels break up into capillaries and the capillaries come into intimate contact with the corresponding maternal vessels. There is no actual intermixture of blood, the gaseous exchange takes place by diffusion through the wall of the capillary. The blood is collected up in the umbilical veins from which it is poured into the inferior vena cava.

The arrangement of the vessels in the placenta differs in different animals. In the rabbit, as shown by Mossman (1926), there is a very beautiful scheme in which the maternal and foetal capillaries lie side by side, and so disposed that the blood entering from the foetus finds itself close to the blood returning to the mother, whilst the blood making its exit to the foetus is in juxtaposition with that arriving from the mother. By this system of vivi-diffusion, the foetal blood can take up oxygen along the whole length of the capillary and emerge the carrier of oxygen at almost as high a partial pressure as that in the arterial blood of the mother.

In most mammalian forms of life the arrangement is different. The maternal blood flows through sinuses of considerable size; in the sheep and goat, the capillaries which contain the foetal blood run in the walls of the sinuses, whilst in the guinea-pig and man these capillaries actually dip down into the sinuses. If the sinus is of considerable size as com-

pared to the capillary, and if the maternal blood in one part of the sinus contains more oxygen than in another, the blood will not emerge from each of the foetal capillaries in a similar degree of oxidation. The net result will be a mixture of blood, some containing more and some less oxygen. This mixture constitutes the blood in the umbilical vein.

It might be supposed that the development of the placenta took place *pari passu* with the need for its utility, that is to say, with the amount of foetal blood which traversed it, or with the amount of oxygen used by the foetus. This is not so. The volume of blood which traverses the foetal heart and the amount of oxygen used by the foetus, as will be shown later, bear a rough relation to the weight of the foetus itself. It is therefore instructive to compare the weight of the foetus with that of the placenta throughout foetal life. In the rabbit it was first shown by Hammond (1935) that whereas the foetus grows progressively, the placenta reaches maximal size earlier, so that if the weight of the foetus was expressed as a percentage of that of the placenta the figure increased progressively. The same is true of the goat and also of the sheep.

Indeed, it seems probable that at the very end of pregnancy the cotyledons shrink. It is not quite clear how this shrinkage affects the efficiency of the placenta as a medium for respiration. It appears to involve the maternal vessels to some extent, and is accompanied by some degree of thrombosis.

The actual size of the capillary bed in the foetal portion of the placenta is not, however, the only factor which regulates the amount of foetal blood which passes through the cotyledons. As pregnancy advances the blood pressure in the umbilical artery becomes progressively higher, as the figures in Table I show.

TABLE I—ARTERIAL PRESSURE IN FOETAL SHEEP

Foetal age in days	49	101	120	123	137	138	140
Pressure in mm Hg	20	34	46	50	72	68	76

It is clear, therefore, that even if the bed remained of constant size the quantity of blood driven through it would increase with the age of the foetus, and consequently the opportunity of taking up oxygen in greater quantities.

It might well be that even if the vascular bed in the placenta did not grow in the later part of pregnancy, and were not extensible, the amount of blood traversing it might be doubled during the last five weeks, as the result of the rise of arterial pressure alone. As the vessels are distensible the rise of blood pressure should more than double the blood-flow.

During this period the foetus approximately trebles in weight, therefore it seems likely that the volume of foetal blood which traverses the placenta bears a fairly constant ratio to the mass of the foetus itself.

What of the quantity of maternal blood which traverses the uterus? Does the volume which irrigates the cotyledons also increase in proportion to the growth of the foetus? We have no information on this subject with regard to the sheep or the goat, but in the rabbit it is quite otherwise. In the rabbit, as in the sheep and goat, the growth of the placenta precedes that of the foetus, and the maternal blood-flow through the rabbit's uterus goes more or less hand in hand with the growth in weight of the placenta.

Lastly it appears that the oxygen used by the foetus bears a fairly constant relation to the weight of the foetus over the last half of foetal life, being in the neighbourhood of 0·0025 cc per gram per minute in the goat.

Thus towards the end of pregnancy, when all the quantities become large enough to be measurable, we have a rather remarkable picture. Schematically, it is as follows: on the foetal side it consists of a rapidly growing foetus, a foetus which irrigates the placenta and takes up oxygen in proportion to the weight of the foetus. On the maternal side, the conditions are relatively stereotyped. The blood-flow does not increase very much nor does the size of the placenta itself. Schematically, therefore, the "oxygen difference" between the blood in the umbilical vein and that in the umbilical artery should remain relatively constant, whilst the maternal blood should leave the uterus increasingly reduced as pregnancy proceeds. That this latter is true in the rabbit is shown in fig. 1. The information is taken from rabbits from which the ovary on one side has been removed and the uterus is therefore pregnant on one side only. A comparison of the blood coming from the uterus on the pregnant side with that which emerges on the other side shows:

(1) That the further pregnancy progresses, the darker becomes the blood emerging from the pregnant side.

(2) That this is not due, as it very well might be, to experimental shock, because the venous blood on the control side becomes if anything brighter. As regards the "oxygen difference" in the foetal blood, the figures given in Table II are available.

The average of the figures in Table II, for the "oxygen difference," is roughly speaking 5 volumes per cent. That of experiment 16 is very different from the rest; over the period which follows it, about three weeks, the foetus nearly trebles in size yet the "oxygen difference" is much the same as the figures before it. If the above picture is correct it follows that

the mean oxygen pressure in the sinuses which contain the maternal blood is dropping throughout pregnancy. As there is no reason to suppose that anything but diffusion accounts for the passage of oxygen from the blood of the mother to that of the foetus—a fact which was shown by Haselhorst and Stromberger (1930, 1931), we may turn to see what factors are

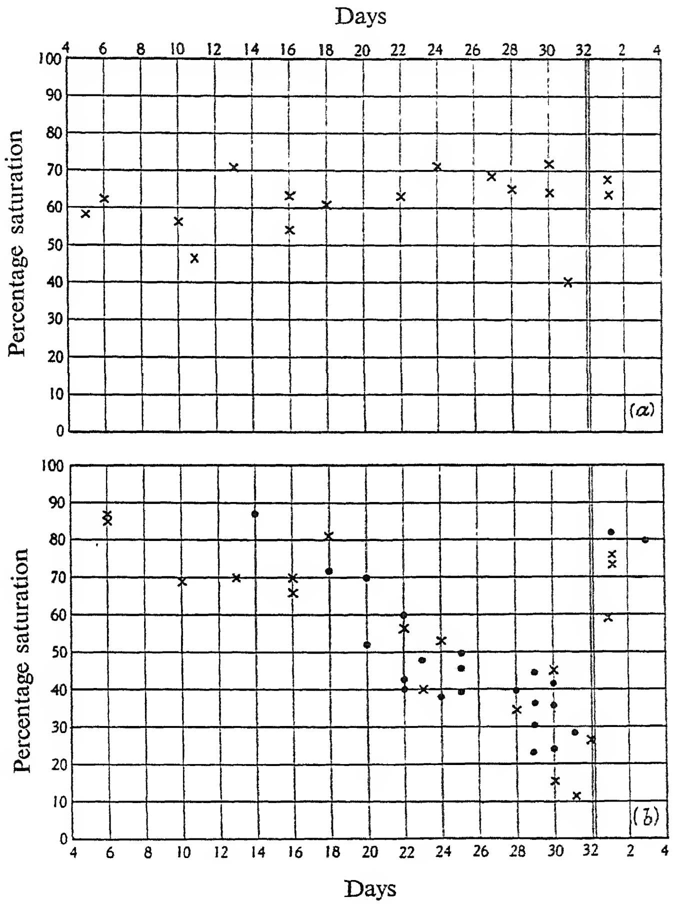


Fig. 1—Percentage saturation of blood emerging from the uterine veins during pregnancy: (*a*) non-pregnant side, (*b*) pregnant side. Ordinate, percentage saturation with oxygen; abscissa, days of foetal life, or after the double line, days after giving birth

involved in the phenomenon. The oxygen breaks away from the haemoglobin of the mother, becomes dissolved in the plasma of the maternal blood and attains a certain partial pressure in that plasma. It then diffuses to the plasma of the foetal blood in which it necessarily exists at a lower partial pressure than that which it set up in the plasma of the

TABLE II

Age of foetus: days	Serial number of sheep	"Oxygen difference" between umbilical vein and artery per 100 cc blood	Weight of foetus in kg
58	2	5·0	0·05
98	8	4·0	0·79
101	50	5·8	0·9
111	11	4·4	1·4
112	14	5·3	1·5
124	16	12·2	1·6
126	17	3·6	2·5
137	19	5·1	3·6
138	27	4·6	3·6
144	29	6·4	4·5

mother. The oxygen then passes to the foetal haemoglobin which it saturates up to whatever point may be possible at the partial pressure in question.

Consider then the membrane between the foetal and maternal bloods. The plasma on either side of it will contain oxygen in solution and at a pressure which will probably be not much greater on the maternal than on the foetal side. We may figure the pressure on the foetal side as x and on the maternal side as $x + a$. Clearly the more oxygen the maternal blood will give up at pressure $x + a$ and the more the foetal blood will accept at pressure x, the greater will be the efficiency of the respiratory process.

Since the arterial blood of the mother is approximately saturated with oxygen, the amount which it will give up at any partial pressure depends upon two factors only, the amount of haemoglobin which it contains per cubic centimetre and the degree to which the haemoglobin becomes reduced at a given pressure of oxygen $(x + a)$, *i.e.*, the nature of the dissociation curve. The steeper the curve in the midde portion and the further it is to the right (according to the conventional delineation in which the oxygen pressure from left to right is the abscissa and the percentage saturation the ordinate) the more readily will the maternal blood yield its oxygen to the foetus, whilst the less inflected the curve of the foetal blood and the further it lies to the left, the more efficient will the foetal blood be for the purpose of taking up oxygen. In the foetal blood also clearly a greater quantity of haemoglobin per cubic centimetre of blood means greater efficiency. Let us then first consider the quantity of haemoglobin in the maternal and foetal bloods; this may be expressed in terms of oxygen capacity.

Fig. 2 shows a number of results which we obtained on sheep. Whilst there is a considerable degree of scatter, amounting to ± 10% of the mean value for the younger foetuses and ± 7% for the older ones, clearly the haemoglobin value of the foetal blood on the whole increases. Incidentally there is one rather interesting determination marked on the figure with a cross. The foetus was one of twins: its fellow was dead and it was feeble. It seems then that there is such a thing as foetal anaemia.

Incidentally the question might arise: has the haemoglobin value of foetal blood any relation to that of the mother? It is known from the work of Scott (1923) that anaemic offspring can be produced by rigid

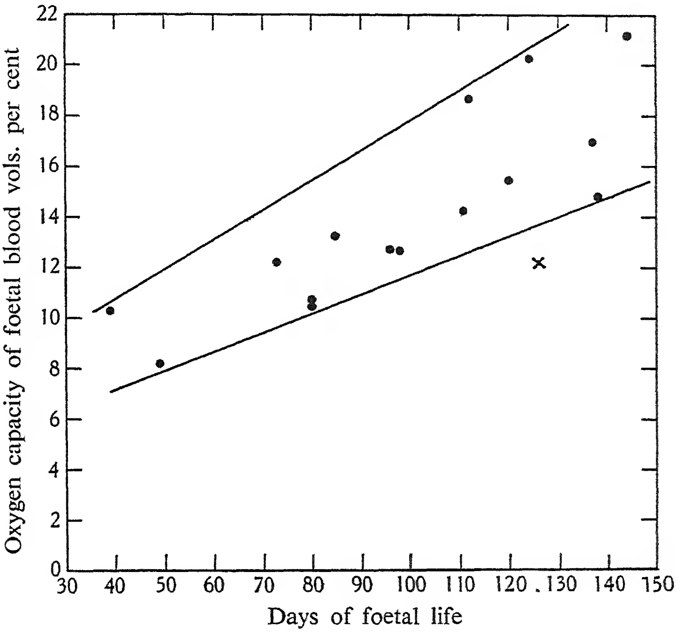


Fig. 2—Oxygen capacity of blood as foetal life progresses

deprivation of iron from the diet of the mother, but in goats presumably normal, if there is any relation, it seems to be in the sense that the greater the haemoglobin value of the mother's blood the less the haemoglobin value of the foetal blood. Fig. 3 shows such data as I have observed.

Now as regards the dissociation curve. From what has already been said, an efficient combination will be for the mother to have such a curve as (*b*), fig. 4, and for the foetus to have such a curve as (*a*); and indeed this is very much what is found. Our information is principally from goats. In these animals the normal dissociation curve of venous blood is within the limits of the dotted lines. Up to about half-way through pregnancy

the dissociation curve in pregnant animals remains in this position, after which it gradually moves to the right until about the 18th of the 21 weeks of pregnancy. The same shift in the curve has been observed independently in man by Eastman, Geiling, and de Lawder (1933); there is complete agreement about the cause, namely, an increase in the hydrogen ion concentration of the maternal blood. Increase of hydrogen ion concentration, however, has its limits, especially in a form so highly developed mentally as man.

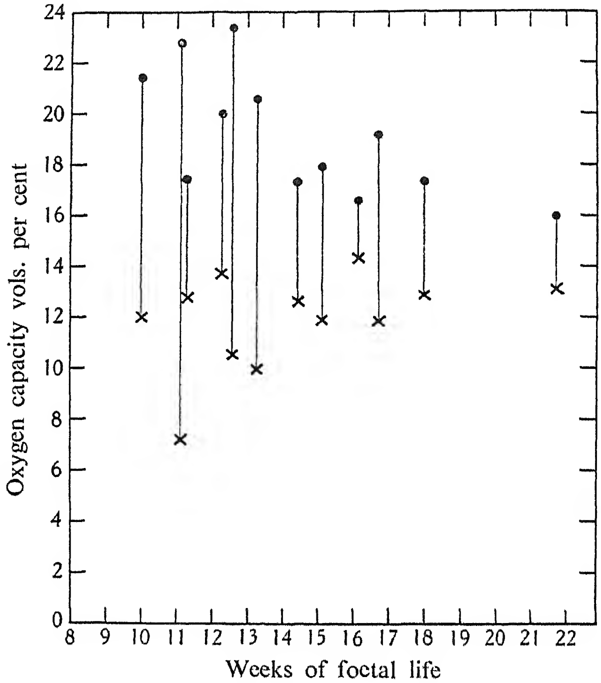


FIG. 3—Comparison of oxygen capacities of maternal and foetal bloods. × foetus; ● mother

The question might arise: can the foetus add to the gap between the mother's dissociation curve and its own by decreasing the hydrogen ion concentration in its own blood? The answer is no, or only to a slight extent. The placental membrane appears incapable of maintaining any considerable difference of p_H between the maternal and foetal plasma. The fact is, therefore, that the foetal plasma, as well as the maternal, is of greater hydrogen ion concentration than is that of the normal animal. Therefore the foetus of the sheep has recourse to a different device. Its blood contains a haemoglobin which is specifically different from that of the mother. The affinity for oxygen is greater and the curve itself is

less inflected. The differences in the nature of the haemoglobin were indicated first by the lesser degree of inflection of the dissociation curve of the blood; but that the haemoglobin itself was fundamentally different in the foetus from that of the mother could only be shown by making preparations of this pigment freed from the corpuscles and in a tolerably pure condition. This was done independently by two workers, McCarthy (1933) and Hall (1934, *a*, 1934, *b*). As they used technique of an entirely

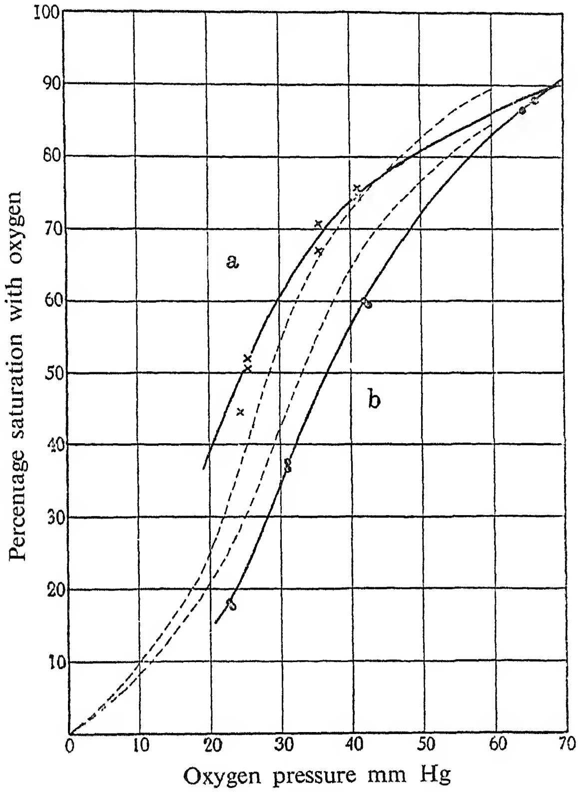


FIG. 4—Typical dissociation curves of (*a*) foetal, and (*b*) maternal blood. Dotted lines indicate limits of normality

different type, the agreement in their findings gave great confidence in the result.

McCarthy purified the haemoglobin by Adair's method and made solutions in considerable amount and of concentrations commensurate with that of the blood. For equilibrium he used the Barcroft saturators and for analysis the Van Slyke pump.

Hall, on the other hand, worked with very dilute solutions which he obtained simply by dropping blood into distilled water. His estimations

were spectroscopic, and he equilibrated his blood in a saturator specially devised for use with the spectroscope.

The discovery of a special sort of haemoglobin in the foetus has naturally opened up a wide field for investigation. What forms of animal life other than the goat (on which the point was first made) have a special form of haemoglobin adapted to the conditions of foetal respiration? This matter has been gone into by Hall (1934, *b*, 1934, *c*), who finds that the rabbit also has a foetal type of haemoglobin, and what is perhaps more extraordinary and extremely interesting is that the same is true of the chick. After all, the problem in the chick is not so very different from that of the foetal mammal. The egg is laid down from the start, with its fixed surface for the entry of oxygen, whilst the embryo is growing the whole time and its demand for oxygen increasing continuously.

Further work undertaken by R. Hill (1935, *a*) and as yet unpublished on the subject of foetal haemoglobin has taken the direction of studying the point at which the alteration to the maternal type takes place. Is it gradual or sudden and what is its nature? Whether gradual or sudden, the curve might be expected to drift from the foetal curve to that of the mother in an orderly way, so that at any time during the change the curve could be analysed.

It might be expected (1) that the dissociation curve of the haemoglobin in a corpuscle once formed would never alter, that at any time during the change 100 corpuscles taken at random would contain $x\%$ foetal haemoglobin and $y\%$ of maternal, the value of x gradually decreasing and of y increasing as time went on; or alternatively (2) that the haemoglobin in the corpuscle would change in such a way that as time went on it became progressively more like the maternal type and therefore that a dissociation curve during the period of change would lie in a position intermediate between that of the foetus and that of the mother, drifting from the former position to the latter.

As regards the time, there seems to be no alteration in the dissociation curve of sheep foetal haemoglobin up to the 137th day.

The black area in fig. 5*a* contains four curves obtained from the foetuses of sheep 1, 8, 10, and 19, respectively 44, 98, 110, and 137 days old, determined under standard conditions, borate buffer $p_H = 9{\cdot}3$, $t = 19°$ C. The area in fig. 5 *b* contains the corresponding curves from the mothers. Two curves obtained from lambs within a few hours of birth (*c*) and (*d*) are shown in fig. 6. They are not intermediate between the foetal (*a*) and maternal (*b*) curves but cross the maternal curve. They are also remarkable in character in that, within the limits of experimental error, it is

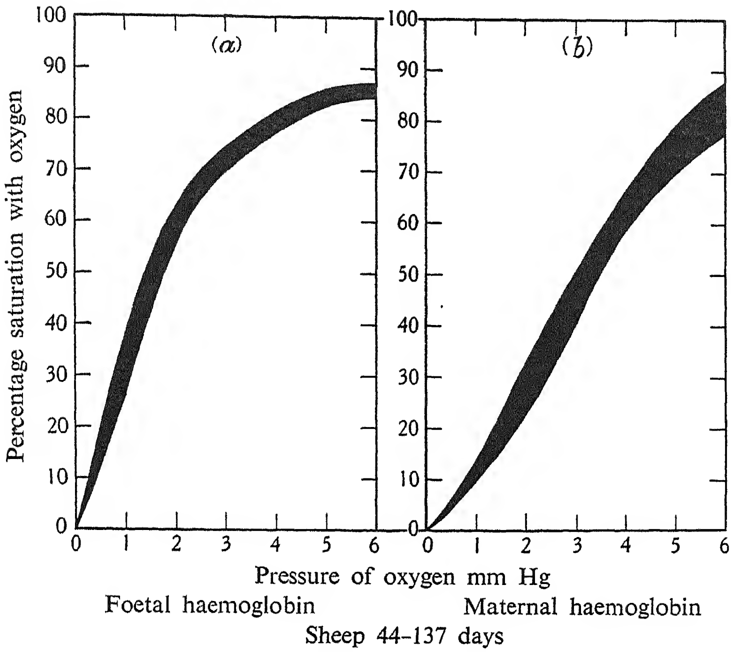


FIG. 5—(*a*) Limits of dissociation curves of foetal haemoglobin. (*b*) Maternal haemoglobin. p_H 9·3, approximate temperature 19° C—R. Hill

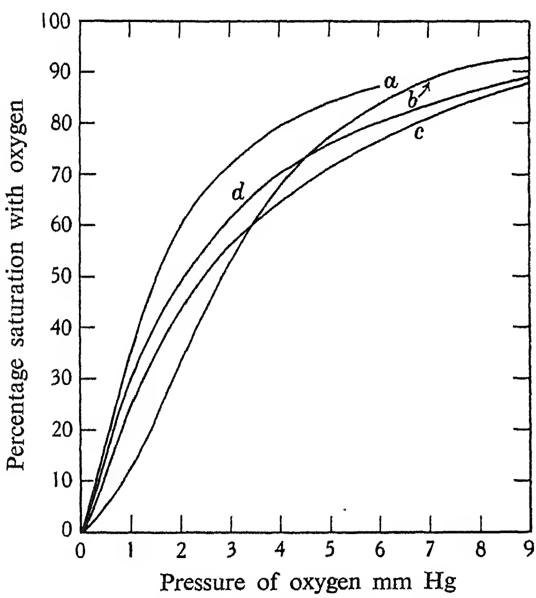


FIG. 6—Oxygen dissociation curves of foetus, mother, and new-born lambs, conditions as in fig. 5. (*a*) foetus (mean); (*b*) mother No. 48; (*c*) lamb (48), 12 hours; (*d*) lamb (49), 12 hours

difficult to say that they show the double inflection common both to the maternal and the foetal types of haemoglobin.

I will not lay any stress on the mathematical shape of these curves. What I do stress is the difficulty of inferring from them the nature of the transition from the foetal to the maternal type. It is not easy to suppose that three different types of corpuscle exist, the foetal, the natal, and the maternal. Nor is it easy to suppose that the haemoglobin changes in the corpuscle in such a way that the natal type is an intermediate stage between the foetal and the maternal. The mystery is frankly unsolved at present.

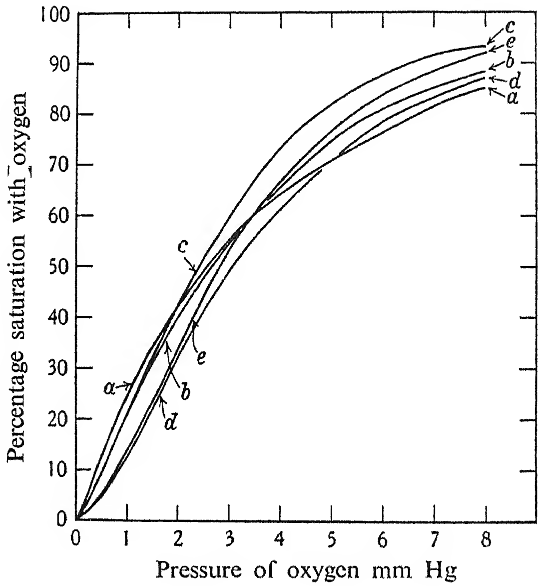


FIG. 7—Oxygen dissociation curves, p_H 9·2—9·3. (*a*) lamb, 12 hours, 19° C; (*b*) lamb, 5·5 days, 21° C; (*c*) lamb, 11·5 days, 18° C; (*d*) lamb, 39 days, 19° C; (*e*) mother, 19° C

By the 11th day after birth, the curve approximates to that of the mother, and by the 39th day it actually falls within the limits shown in fig. 5*b*, *see* fig. 7.

Let us turn to another phase of the subject, which may or may not have some ultimate bearing on the matter.

Quite recently Haurowitz (1935) published a series of curves which showed the difference between the haemoglobins of the mother and the new-born child. These curves were remarkable in showing that the haemoglobin of the baby had a less affinity for oxygen than that of the mother. This is the direct opposite of what I have shown you in

the sheep and the goat, the rabbit, and the fowl, and is apparently unphysiological.

Working on samples of human foetal blood sent by Professor Fleming from the Obstetrical Department of the Royal Free Hospital, R. Hill (1935, *b*) independently observed the same phenomenon in human foetal blood, thus to that extent extending and confirming the work of Haurowitz. Hill proceeded to compare the dissociation curve of the corpuscles of the foetus, suspended in a buffer solution of p_H 7·4, with that of a similar suspension of the corpuscles of the mother. He found that there was a reversal of the positions of the dissociation curves, and that under the conditions of the experiment, the human foetal haemoglobin in the corpuscles has a greater affinity for oxygen than the maternal haemoglobin also contained in the corpuscles.

In the sheep the curve of the foetal haemoglobin and that of the maternal haemoglobin are shifted to the right to about the same extent, when the haemoglobin is packed into corpuscles. In man, the shift for the foetal haemoglobin is about the same as that in the sheep, but the shift for the maternal curve is much greater. The following criticism demands some consideration. The position of the normal dissociation curve derives significance from the fact that whilst it affords ample opportunities for oxygen to be picked up by the blood in the lung, it also relinquishes the oxygen at a partial pressure high enough to impart oxygen efficiently to the tissues. The foetal haemoglobin, whilst it can pick up oxygen with considerably greater facility, parts with it with corresponding difficulty. Is there not a prospect of some degree of asphyxia of the tisues? In answer to the above criticism I may say at once that the tissues of the foetus use much less oxygen than those of the mother, but I must justify the statement.

I turn therefore to the consideration of the basal metabolic rate of the foetus. This cannot be expressed in the usual way as calories per square metre of surface, because in the metabolic sense the foetus has no surface, that is it has no cooling surface exposed to the outer air. We must content ourselves with the measurement which Chauveau and Kaufmann used to call the coefficient of oxidation—the average number of cubic centimetres of oxygen used per minute by each gram of foetal tissue.

This measurement has been attempted at various times and by various workers. Zuntz and Cohnstein (1884) tried to do it by measuring the difference between the oxygen in the arterial and venous blood in the umbilical vessels of the sheep, and multiplying the oxygen difference by the rate of blood-flow which they also measured. The latter measure-

ment was made with a stromuhr. The sheep, unlike man, has four umbilical vessels. Zuntz and Cohnstein obtained low and variable results. Their method never seemed to me a practicable one. The umbilical vessels are very sensitive to any interference, even the insertion of a hypodermic needle. They tend to " seize up " to such an extent that cannulation almost means obliteration. To put a stromuhr in any one vessel would probably divert the blood to a parallel route. To put stromuhrs either into both arteries or into both veins would probably cut down the blood-flow, as indeed it seems to have done in Zuntz and Cohnstein's experiments.

Christian Bohr (1900) attempted to measure the oxygen used by foetal guinea-pigs by difference, *i.e.*, he measured the metabolism of the pregnant guinea-pig, then pinched or ligatured the umbilical cord and then repeated the measurement, and by subtracting the second measurement from the first arrived at a figure which purported to be the oxygen used by the foetal guinea-pigs.

I have a deeply rooted aversion, it may be prejudice, against this type of method. Even in ordinary physical methods, the estimation of a quantity as the difference of two much larger quantities is a process not to be recommended. In animal experiments of this type the method assumes a degree of constancy on the part of the mother that is very difficult to justify.

My colleagues, Flexner and McClurkin, and I (1934) worked on the goat. The basis of our measurements consisted in:

(*a*) A phethysmographic measurement of the amount of blood which traverses the foetal heart.

(*b*) Certain blood-gas measurements which on the one hand give us the amount of oxygen taken up by each cubic centimetre of blood which traverses the placenta and, with the help of certain assumptions, give us the probable proportion of (*a*) which is involved in the placental circulation.

We do not claim anything but a rough approximation, but, such as it is, it gives rather surprising results. During the later half of foetal life:

1—The ratio of the blood-flow through the foetal heart to the weight of the body varies little. It is of the order of 0·12–0·18 cc per gram of foetus per minute.

2—The quantity of blood which traverses the foetal heart is, relatively to the body weight, but little less than that of the adult.

3—The oxygen used by the foetus also remains constant during the later part of foetal life and is about 0·0025 $\pm$ 0·001 cc per gram per minute.

4—The oxygen used by the foetus, relatively to the body weight, is only a fraction, perhaps a quarter or a fifth, of the post-natal value.

Firstly then let me emphasize the contrast between the blood-flow which in volume alters little at birth, and the oxygen consumption which increases several-fold.

I said that these calculations were made as a very rough approximation and with the help of certain assumptions. Let me now dwell for a moment upon the assumptions in question. They reduced the foetal circulation to the simplest possible terms, frankly ignoring the teaching that the blood which goes to the head is redder than that which goes to the body; the arterial blood was taken as being of the same composition to all organs, namely the composition of the blood in the umbilical arteries. The second assumption made was that the blood in the great veins was completely reduced. The reason why these assumptions were made was that the data for the calculations were simply the oxygen content and percentage saturation of the blood in the umbilical artery and vein and the volume of blood circulating through the heart. There was also the assumption that the volume of oxygen picked up by the blood in the placenta was the same as that used in the body of the foetus.

In some of the experiments rather more data were available and quite different assumptions were made. Of these the first was that the blood in a carotid artery was the most arterial available and found its way there without intermixture with venous blood from the body; the second was that the "oxygen difference," whether in the head or the body, was 5 volumes per cent. The first set of assumptions gave, in four experiments in the later half of pregnancy, figures ranging between 0·0016 cc and 0·0035 cc of oxygen per gram of foetus per minute. The second set of assumptions gave for two experiments 0·0021 and 0·0025 cc. It seems, therefore, that though the assumptions made were quite different in the two cases the results came out about the same.

Nevertheless it seemed unsatisfactory not to test the assumptions when the opportunity came for doing so. Of these assumptions the most interesting are those connected with the mixing of blood in the heart.

The classical description of the foetal circulation teaches that some or all of the blood which arrives by the inferior vena cava, the most arterial available to the foetus, is projected through the foramen ovale into the left auricle, passes to the left ventricle, thence to the arch of the aorta, and so in part along the carotid arteries. The blood from other vessels, the superior vena cava and presumably the azygos vein, passes from the right auricle to the right ventricle, then up the pulmonary artery and in

part via the *ductus arteriosus* to the aorta. There it mixes with such of the aortic blood as has not already been diverted to the head and this mixed blood, less red than the supply to the head, supplies the body. What support do actual blood-gas determinations give to this description?

To take the simplest comparison first. To what extent, if at all, does the blood in the carotid artery differ from that in the systemic aorta?

TABLE III

Serial No. of sheep	Foetal age: days	Percentage saturation of blood with oxygen: Carotid	Aorta (umbilical artery)	Difference
11	111	72	48	24
14	112	64	64	0
16	124	41	23	18
19	137	22	10	12
27	138	50	43	7

Except with sheep No. 14 the blood in the carotid contained more oxygen than that in the aorta, in three animals much more. The anatomical findings therefore seem to be substantiated. Evidently the blood in the aorta contains a considerable intermixture of dark blood from the veins of the head, with the bright blood from the inferior vena cava. I use the term veins from the head for convenience—it includes, however, the veins from the arm, the azygos veins and, what is very important, the coronary veins; in short everything but the inferior vena cava. I determined to investigate the matter further, and started doing so on the assumption that the blood from the jugular vein might be taken as representing the whole of the blood from the head.

The blood from the umbilical artery was taken as typical of that from the aorta (below the entry of the *ductus arteriosus*).

The observations given in Table IV shed light on the constitution of the mixture in the aorta.

I was faced with the astonishing result that in three out of four experiments in sheep and in one goat the blood in the aorta contains less oxygen than that in the jugular vein. This can only mean that the blood in the jugular vein is not typical of the "blood from the head," that it is redder than that from the arms, etc., but also it leaves little room for admixture with blood from the inferior vena cava. Unless the above figures are entirely erroneous we must conclude that the blood in the aorta is almost pure "venous blood from the head" and that in fact the crossing so far as the body is concerned is almost complete.

If the blood in the abdominal aorta approximates to that of the veins from the upper part of the body, to what extent does the blood in the carotids approximate to that in the inferior vena cava?

TABLE IV

No. of sheep	Foetal age: days	Percentage saturation of blood with oxygen: Thoracic inferior vena cava	Jugular vein	Umbilical artery
11	111	70	39	48
16	124	56	33	23
19	137	26	16	10
29	138	48	41	18
Goat 32	115	—	32	19

The figures in Table V suggest that if the blood which reaches the heart from the inferior vena cava is not diluted to a great extent, on its way through the heart to the head, we are then left in the following position:

(1) in the passage of the blood through the heart there is almost complete crossing of the streams;

TABLE V

Serial No. of sheep	Foetal age: days	Percentage saturation of blood with oxygen: Thoracic inferior vena cava	Carotid
11	111	70	72
14	112	73	64
16	124	56	41
19	137	27	22

(2) the blood in the carotid shows that there is some, though not much, addition of venous blood from the head to the red blood from the inferior vena cava;

(3) as regards the blood in the abdominal aorta, there is probably some, though not much, addition of blood from the thoracic aorta to the "venous blood from the head";

(4) if the above be accepted it follows that the two streams are of approximately equal volume, half the blood leaving the heart going to the head and half to the lower part of the body and the placenta.

These results might seem somewhat academic and such as might be complacently set aside for future enquiry but for the fact that the last, which follows from those which precede it, involves certain rather important implications. The first is that roughly half the blood which leaves the heart does so through the *ductus arteriosus*. The second is that the pressure in the right ventricle must be approximately the same as that in the left. The third follows on the heels of the first. If the *ductus arteriosus* plays so important a part up to the end of foetal life, in virtue of what mechanism is it approximately closed at birth? To the *ductus arteriosus* we shall return. We are then dealing with the assumptions which were made in certain experiments, in which we measured the coefficient of oxidation, and we have arrived at the point of saying that the blood leaving the heart divides into two approximately equal streams, one of which goes to the lower part of the body and includes the placental circulation; the placental circulation must therefore be less than half the total blood leaving the heart. It will be remembered that in one set of assumptions we called it one-third of the whole circulation, in the other we made no assumption about the ratio of the placental circulation to that of the body.

TABLE VI

Serial No. of sheep	Foetal age: days	Percentage saturation of blood with oxygen		
		Umbilical vein	Abdominal inferior vena cava	Thoracic inferior vena cava
11	111	79	$<48, >0$	70
14	112	82	19	73
16	124	79	$<23, >0$	56
19	137	42	$<10, >0$	27
29	138	61	$<19, >0$	48

The question now is: can we arrive at any view as to what proportion of the blood in the abdominal aorta goes to the placenta and what proportion goes to the rest of the body? There seems no possibility of doing that on the arterial side; but there yet remains the possibility of studying the confluence of the bloods from the umbilical vein and the abdominal inferior vena cava to form the thoracic inferior vena cava.

We have very few analyses of the blood from the abdominal inferior vena cava, but clearly that blood must contain less oxygen than the blood in the abdominal aorta and more than nothing (at least no analysis has given blood from this situation absolutely devoid of oxygen). The figures which we have at our disposal are given in Table VI.

From these figures it is clear that the great bulk of the blood returning to the heart from the lower part of the body + the placenta comes from the placenta and not from the body. The situation is, however, not so simple as the description just given might indicate, for just at the junction of the umbilical vein with the abdominal vena cava lies the liver. In the ruminants the umbilical vein divides; one part goes as the *ductus venosus* directly into the vena cava between the hepatic veins and the heart, the other goes into the portal vein, which means that the blood brought by the umbilical vein from the placenta traverses the capillaries of the liver. In some animals, *e.g.*, the horse, there is no *ductus venosus*, and the whole of the blood from the placenta goes into the portal circulation. It is remarkable that the accounts given of the foetal circulation do not stress the physiological significance of this arrangement. The liver is, in fact, the most favoured organ. It receives the great mass of its blood direct from the placenta, *i.e.*, from the locality where the blood is acquiring the essential ingredients (including oxygen) for the nutrition of the foetus. Functionally speaking, the placenta is at once the alimentary canal and the lung of the foetus: on the blood from it—in the horse, on all the blood from it—the liver has a first option. Comparing the circulation in the foetal liver with that in the adult liver, the umbilical blood possesses the metabolic properties of the blood both of the adult hepatic artery and of the adult portal vein.

This is, however, a digression; our immediate point is that the blood in the thoracic vena cava resembles that in the umbilical vein much more closely than it resembles that in the abdominal vena cava and therefore that in volume the umbilical blood altogether preponderates in the mixture. The considerations which I have put forward about the liver tend to make the calculated preponderance even greater. It would seem difficult on this figure to suppose that more than one-third of the blood in the thoracic vena cava comes direct from the body of the foetus and less than two-thirds from the placenta. Possibly the disproportion is even greater.

Summing up, therefore, we are left with the following general picture: that of 300 cc of blood which traverse the foetal heart, about 150 goes to the head and 150 to the abdominal aorta; of the latter 150, perhaps 100 goes to the placenta for aeration and 50 to the body of the foetus for the nourishment of the same.

Can this be? Can the disproportion between the head and the body be so great as that 150 cc should go to the former and 50 cc to the latter? Moreover, 150 cc of good blood to the head, and to the body 50 cc of blood most of which has already done the tour of the head. The answer

to this question I will take up shortly, but at the moment we seem to have arrived at the general position that provisionally the blood-flow through the placenta may be taken as about a third of that through the heart and that the blood-flow through the heart may be taken as about 0·15 cc per gram of foetus per minute, and the oxygen used per gram per minute may be found by comparing the blood in the umbilical artery and vein; finding the oxygen difference between these per cubic centimetre of blood and multiplying it by 0·05. The result is given in Table VII.

TABLE VII

Serial No. of sheep	Foetal Age: days	Weight kg	Oxygen in cc per 1 cc blood: Umbilical vein	Umbilical artery	Difference	Oxygen used per gm of foetus per min
2	58	0·05	0·078	0·028	0·05	0·0025
8	98	0·79	0·088	0·048	0·04	0·0020
11	111	1·4	0·113	0·069	0·044	0·0022
14	112	1·5	0·157	0·104	0·053	0·0027
16	124	1·65	0·170	0·052	0·122	0·0061
19	137	3·6	0·068	0·017	0·051	0·0026
27	138	3·6	0·105	0·059	0·046	0·0023
29	144	4·5	0·102	0·038	0·064	0·0032

With the exception of sheep 16 the coefficients all fall between 0·0020 and 0·0032 or 0·0026 ± 0·0006 cc per gram per minute.

The oxidation coefficient 0·0025 cc per gram per minute may be compared with that of the whole animal. This in adult goats is known from the extensive observations made at Porton (Barcroft, Boycott, Dunn, and Peters, 1919), and for resting animals is on the average 0·0085 cc per gram per minute, whilst in anaestheticized kids it varies from 0·011 to 0·005 cc.

For sheep the figure given by Reisel* is 343 cc of O_2 per kg per hour or 0·0057cc per gram per minute, and in Smith's "Veterinary Physiology" it is given as 32·5 cubic feet of oxygen in 24 hours for a sheep of 99 lb, which works out to the much higher figure of 0·028 cc of oxygen per gram per minute.

Thus the degree of respiration of the foetus before it is born is only a fraction of its value after birth. We may dwell for a moment on the reason. Practically none of the foetal tissues is active, with the exception of the liver and the heart. The kidneys, the central nervous system, the

* *See* Schafer, "Text Book of Physiology," vol. 1, p. 707.

muscles, the digestive glands are all at rest. The nutritive and excretory functions are performed by the mother. So far as heat production is concerned the foetus has no cooling surface of its own, and so far as its presence increases the cooling surface of the mother doubtless the responsibility for extra heat formation is borne by the mother itself. On grounds of heat formation, then, there is no reason to maintain the muscular tone of the foetus and indeed it is not maintained. How closely muscular tone and cutaneous activity are related at this stage may be shown by the following experiment, records from which are shown in fig. 8, Plate 14. They were taken by Dr. Barron (1935). The foetus being completely immersed in the saline bath (the cord, of course, being intact and the foetal circulation satisfactory), electrodes are thrust into various muscles for the purpose of tapping the muscle currents. There is silence—indicating that individual fibres are not contracting. The foetus is now lifted from the bath on to a shelf just above it, care being taken not to stretch the cord. The muscle currents at once appear. They are abolished by putting the foetus back into the bath.

So long, therefore, as the skin is not exposed there is no muscular tone and the only contraction of the fibres is that contingent upon the occasional muscular movements.

The nervous system, too, is in a very quiescent condition; to go into that in detail would require a lecture in itself: here I will only say that by the 92nd day of foetal life in the sheep it is possible to elicit nearly all the reflexes of which the lamb is capable except those which have to do with gravity. Not only so, but it is much more easy to elicit them at that stage than later. I will take one particular activity of the central nervous system as an example because it is so easy to demonstrate, namely, the gasp.

At 49 days spontaneous respiratory movement was observed. At 68 days it persisted, but also there were immediate respiratory movements both on compressing and releasing the cord.

The least interference with the cord will provoke respiratory movements in a foetus 80 days old within 10 seconds; by the 92nd day the cord must be occluded for 16 seconds before the respiratory spasm takes place, whilst in a foetus of 138 days over a minute will elapse from the moment of occlusion of the cord until the central nervous system is so quickened in activity as to produce a gasp—but now I am passing from the physiology of the foetal life to that of birth.

I can now gather up one or two points which, not to break my argument, I had deferred for discussion. If this picture which I have drawn is correct; if there is almost complete crossing of the blood-streams in the

heart, if one-half of all the blood goes from the left ventricle along the aorta to the head and the other half goes along the *ductus arteriosus*, two-thirds of it to the placenta and one-third to the body, certain things must almost inevitably follow.

Firstly the term " head " must embrace a great part of the whole organism.

Secondly the *ductus arteriosus* must be capable of allowing a great amount of blood to pass along it.

These two points have been tested from a negative point of view. Firstly in the guinea-pig foetus, the aortic arch was tied just proximally to its junction with the *ductus arteriosus*, the left ventricle was then cannulated and blue injected into it. The blue, which, of course, could not get

TABLE VIII—FOETAL AGE 143 DAYS

Perfusion pressure cm blood pulmonary artery	Outlet pressure cm blood aorta	Blood traversing *ductus arteriosus* cc per min
32	0	45
46	0	59
61	0	83
82	0	120
103	0	156
103	44	98
103	58	88
103	69	66
Aorta	Pulmonary artery	
103	0	136 130
103	69	86 70

beyond the ligature, reached not only the brain but the spinal cord as far down as the top of the lumbar swelling, both arms, of course, the whole of the chest wall, and the heart; the only muscular organs actually active. When it is borne in mind that the liver receives its blood supply from the placenta, there seems very little that is of importance to which the abdominal aorta is the sole supply, the alimentary canal which is not used, the kidneys, which also are not at this stage essential organs of secretion, and the muscles of the lower limbs and the lower part of the trunk which are flaccid. Here I would express a caution; the above injection does not inform us of what does happen, merely what can happen. The point is that had the injection showed blue only in the

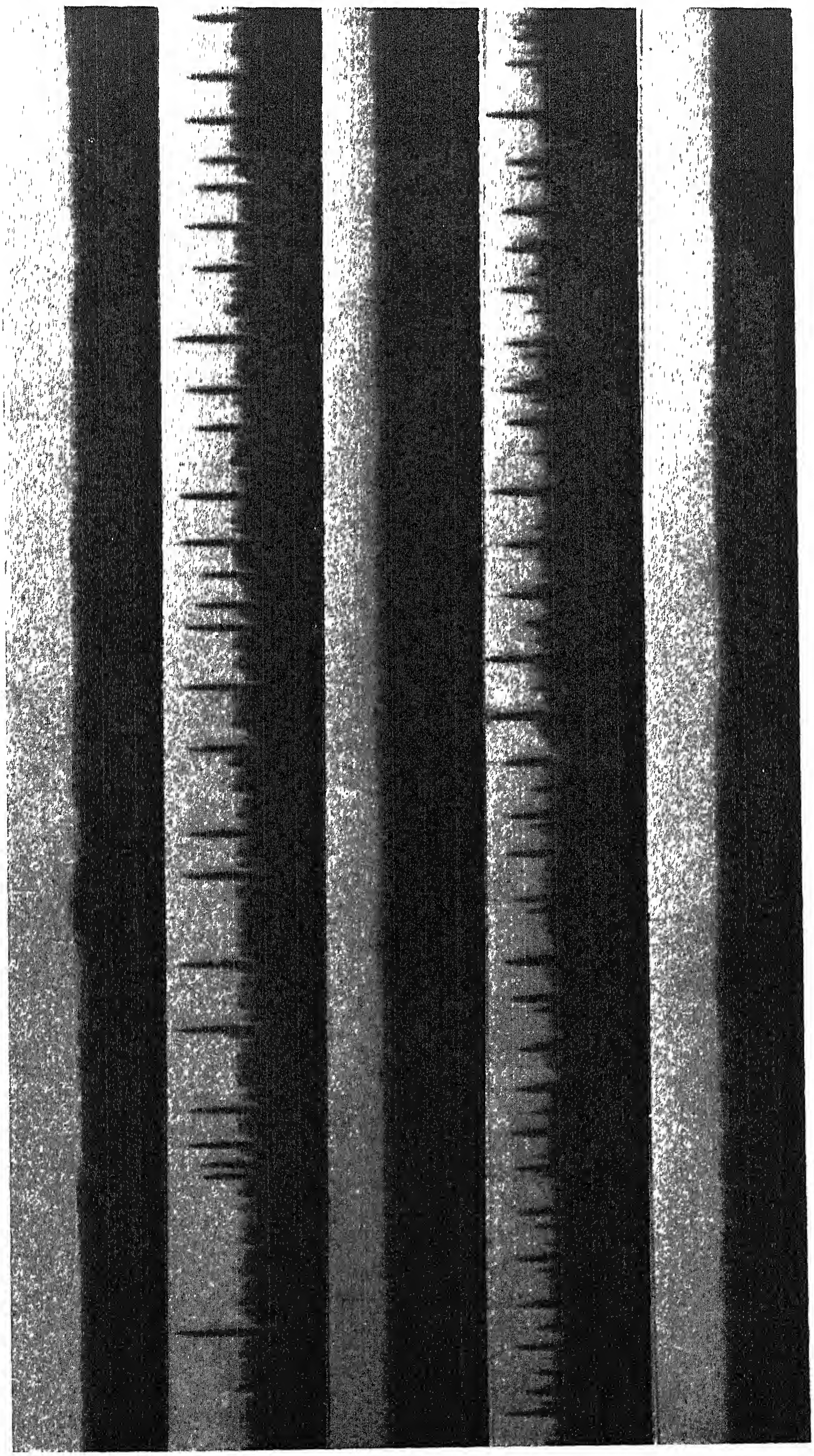


Fig. 8—Sheep 30, 144 days. Record of muscular tone in foetus of sheep, respiration maintained through umbilical cord (Barron, 1935) *a*, *c*, and *e*, foetus in bath; *b* and *d*, foetus out of bath.

actual head and arms it would have been difficult to suppose that three times as much blood was supplied to them as to the body.

As regards the *ductus arteriosus*, if it were shown by perfusion that only a trickle of blood could pass along the *ductus arteriosus*, it would be futile even to consider the possibility of something approaching half the output of the heart being driven along it.

Some perfusions were therefore carried out. These were with cold blood and in the dead, recently alive, vessel.

To find that so great a volume of blood can find its way through the *ductus arteriosus* eases the conception of the crossing of the blood-streams during foetal life, but if it helps to settle that particular point it immediately raises another: how is this so great flow stopped at birth so as to produce an immediate transition from the pre-natal to the post-natal circumstances of circulation? Here again I am drifting into the physiology of birth, and here I shall leave the subject.

References

Barcroft, J., Boycott, H. B., Dunn, J. S., and Peters, R. A. (1919). 'Quart. J. Med.,' vol. **13**, p. 35.

Barcroft, J., Flexner, L. B., and McClurkin, T. (1934). 'J. Physiol.,' vol. **82**, p. 498.

Barron, D. H. (1935). *Unpublished.*

Bohr, C. (1900). 'Skand. Arch. Physiol.,' vol. **10**, p. 413.

Eastman, N. J., Geiling, E. M. K., and De Lawder, A. M. (1933). 'Johns Hopkins Hosp. Bull.,' vol. **53**, p. 246.

Hall, F. G. (1934, *a*). 'J. Physiol.,' vol. **80**, p. 502.

—— (1934, *b*). 'J. Physiol.,' vol. **82**, p. 33.

—— (1934, *c*). 'J. Physiol.,' vol. **83**, p. 222.

Hammond, J. (1935). *Unpublished.*

Haselhorst, G., and Stromberger, K. (1930). 'Z. Geburtsh. Gynak.,' vol. **98**, p. 49.

—— (1931). *Ibid.*, vol. **100**, p. 48.

Haurowitz, F. (1935). 'Hoppe-Seyler's Z.,' vol. **232**, p. 125.

Hill, R. (1935, *a*). *Unpublished.*

—— (1935, *b*). *Unpublished.*

McCarthy, E. F. (1933). 'J. Physiol.,' vol. **80**, p. 206.

Mossman, H. W. (1926). 'Amer. J. Anat.,' vol. **37**, p. 433.

Scott, J. M. D. (1923). 'Biochem. J.,' vol. **17**, p. 157.

Zuntz, N., and Cohnstein, J. (1884). 'Pflügers Arch.,' vol. **34**, p. 173.

576 . 31

The Effect of Ultracentrifuging on the Cells of the Root Tip of the Bean (*Phaseolus Vulgaris*)

By H. W. Beams and R. L. King, Department of Zoology, State University of Iowa*

(*Communicated by J. P. Hill, F.R.S.—Received February* 15, 1935)

[Plates 15–18]

In several recent papers (Beams and King, 1934, *a*, *b*; 1935; Beams, Muliyil and Gatenby, 1934) we have reported results on the effects of ultracentrifuging various animal somatic cells. We have shown that many of the cytoplasmic and nuclear materials could be readily displaced and stratified into distinct regions according to their relative specific gravity. Up to this time practically no work had been done on centrifuging animal somatic tissues with the exception of that on blood. The main deterrent in this type of investigation seems to have been the lack of sufficient centrifugal force available to displace materials of relatively little difference in specific gravity in these highly viscid cells.

The results of our studies on ultracentrifuging animal cells seem to warrant the application of this technique to the study of plant tissues. Accordingly, we have used it as an experimental method, supplementing the usual cytological procedures in an attempt to cast some light on the debated and much confused problems concerning the structural elements of plant cytoplasm.

Material and Methods

The material for this study consists of root tips of the bean (*Phaseolus vulgaris*), chosen mainly because it has served as a partial basis for rather extensive investigations on the cytoplasmic components by Bowen (1928, 1929). The beans were germinated in a moist chamber, and when the roots were about one-half inch long they were snipped off and placed in the rotor of an air-driven ultracentrifuge similar to that recently described by Beams, Weed, and Pickles (1933).† They were then centrifuged in water, at approximately 400,000 times the force of gravity, for

* The senior author wishes to thank Professor J. B. Gatenby of Trinity College, Dublin, for the facilities of his laboratory during the preparation of the manuscript.

† We wish to take this opportunity to thank Professor J. W. Beams and Mr. A. J. Weed of the University of Virginia for kindly constructing the ultracentrifuge for us.

15 to 20 minutes and subsequently prepared by the osmic impregnation method of Kolatchev and that of Weigl, as recommended by Bowen (1929, *b*), for the demonstration of the osmiophilic platelets. Others were fixed in Regaud's or in Champy's fluid and stained in iron-haematoxylin for the study of other structures in the cell. All material was embedded in paraffin and cut into sections 4 to 8 microns in thickness.

Nomenclature

On reading the recent literature concerning the structural elements of plant cytoplasm, we immediately find ourselves in the midst of what certainly seems like confusion. However, we do not wish to enter into a long discussion of this literature here since the subject has been adequately reviewed and discussed in a number of papers by Dangeard (1933), Bowen (1928, 1929, *a*), Guilliermond (1929, 1932), and others. After considerable deliberation we have decided to adopt, in general, the terminology used by Bowen; not that we believe it completely satisfactory, but because it seems more convenient for our purpose since we are working on similar material. Accordingly, we have found in the cytoplasm of the bean root tip a number of materials and structures which are more or less readily separated on the basis of morphology, tinctorial reaction, and specific gravity. The entities thus demonstrated may be listed as follows:

1—Nucleus, including chromatin, nucleolus, and nuclear sap.
2—Osmiophilic platelets, according to the conclusion of Bowen homologous to the Golgi apparatus of the animal cell.
3 (*a*)—Plastidome, including the elongated proplastids and plastids, homologized by Weier with the Golgi apparatus of the animal cell.
3 (*b*)—Starch granules.
4—Pseudo-chondriome, generally accepted as homologous to the chondriome of the animal cell.
5—Vacuome, thought to be homologous to the Golgi apparatus of animal cells by Guilliermond, Parat, and others.

In our description we shall follow this order and the diagram of the general anatomy of the angiosperm root-tip given by Bowen (1929, *a*) to indicate, as far as possible, the general region from which the cells figured were taken.

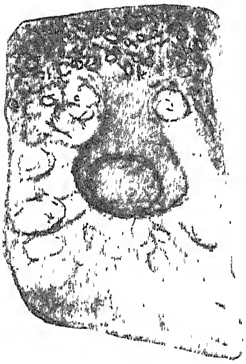
1—*Nucleus*

It will be immediately noted upon examination of the illustrations of this paper that the most striking effect of centrifugation is the displace-

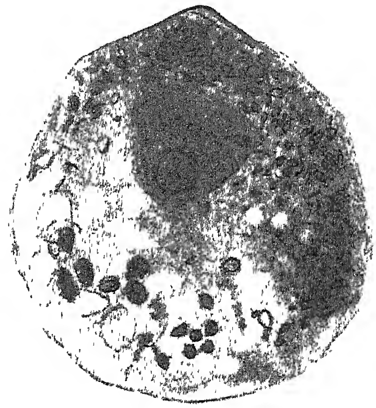
ment of the nucleolus to the centrifugal pole of the nucleus where it usually rests in contact with the nuclear membrane. In some cases the nucleolus may even be forced completely through the nuclear membrane. Similar results have been obtained by Mottier (1899), by Andrews (1915), and by Nemec (1929), but displacement of the nucleolus was not observed by Kostoff (1930) or by Luyet and Ernst (1934). Chromatin is generally moved centrifugally along with the nucleolus. In some cells the nucleus itself appears to be displaced but little, figs. 3, 13, and 15, Plates 15 and 17, while in others it shows a marked centrifugal displacement, figs. 4, 10, 22, and 24, Plates 15, 16, and 18. In figs. 1 and 5, Plate 15, an elongation of the nucleus has occurred; here it appears as though the nuclear sap is less dense than the cytoplasm and is being forced to the centripetal pole. Luyet and Ernst (1934) have figured the complete cleavage of the nucleus into a lighter part, consisting of the nuclear sap, and a denser part containing chromatin and nucleoli in cells centrifuged at 30,000 g. The position of the nucleus in centrifuged cells seems to depend largely upon the degree of development of other elements present. Thus, when plastids and starch grains are present in small numbers, fig. 4, the nucleus moves centrifugally because it is heavier than the cytoplasm; but when they are present in large numbers, figs. 6, 21, and 23, Plates 15 and 18, the nucleus moves centripetally, because it is lighter than these elements. When the vacuole is well developed, figs. 19, 20, 22, and 24, Plates 17 and 18, the nucleus, being heavier than the vacuole, moves centrifugally.

2—*Osmiophilic Platelets*

The late Professor Bowen (1927, *a*, 1928, 1929, *a*) has described in plant cells what he claimed to be a " new category of cytoplasmic elements, hitherto unnoticed by botanists, because they can, as a rule, be demonstrated only by osmic acid impregnation." He suggested that they were the homologue in plant cells of the Golgi bodies in animal cells. However, as will be discussed elsewhere, Bowen's views have not been widely accepted in many quarters. Nevertheless, we wish to say at the beginning that whatever category in the cell to which one assigns the osmiophilic platelets, the fact remains that Bowen's figures of them are substantially correct. It is admittedly a very difficult task to determine the exact morphology of these bodies, mainly because of their small size, but the impression one gets of them is that of small discs or plates, the periphery of which is intensively darkened by osmic acid. When seen in face view their darkened rims give them the appearance of small rings; in profile they frequently appear as rods and ellipses of various degrees depending



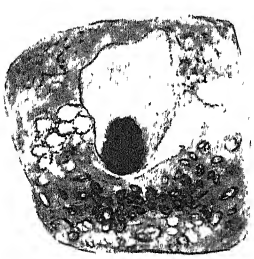
1



2

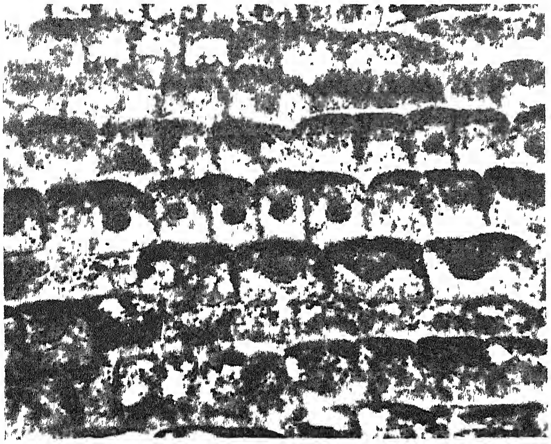
3

4



5

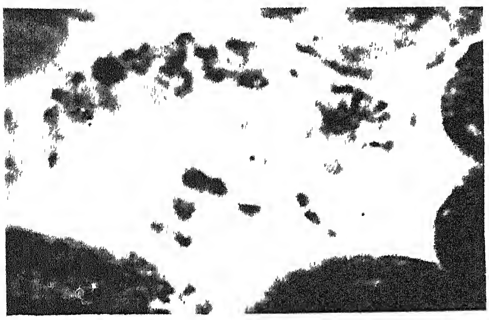
6

7


8


9


10

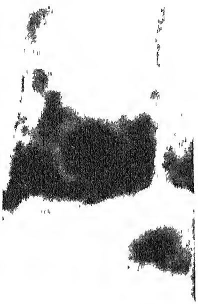


11

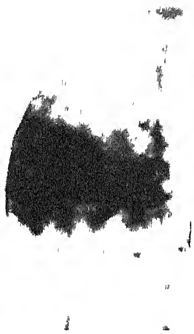

12


13


14


15


16

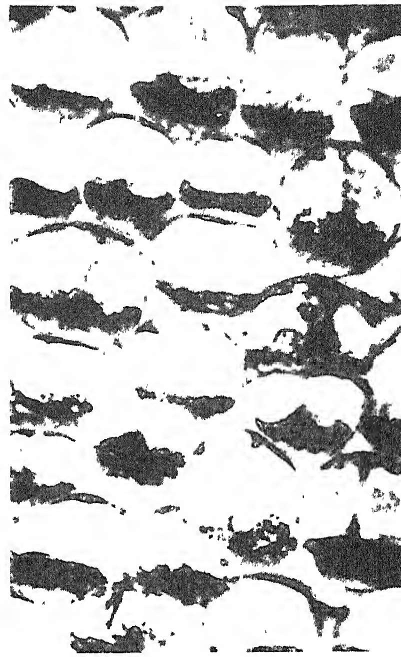

17


18


19

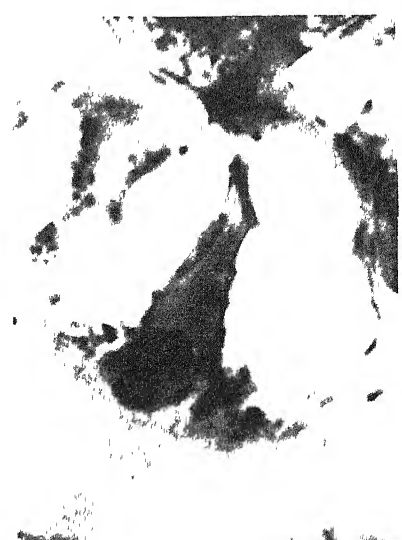

20

21



22

23



24

upon the angle of view. In fact, they bear a striking resemblance in many ways, which will be discussed below, to the Golgi bodies of many insect tissues. The osmiophilic platelets are generally numerous and in uncentrifuged cells are evenly distributed throughout the cytoplasm. (*See* Bowen, 1928, for a detailed description of osmiophilic platelets.) In ultracentrifuged root tips of the bean prepared by osmic acid methods the osmiophilic platelets are clearly seen displaced to the light or centripetal pole of the cells. In fig. 7, Plate 16, is shown a low-power view of a considerable region of the periblem of the root-tip, just above the root-cap, after such treatment. Figs. 1 and 8, Plates 15 and 16, are high-power views of the same type of cells. It is interesting to note that the osmiophilic platelets, like the Golgi bodies in Helix (Beams, Muliyil, and Gatenby, 1934), still retain their general form after being forced through the cytoplasm to the centripetal pole of the cells. The nuclei in these cells are elongated in the direction of the centrifugal force with the nucleolus forced to the centrifugal pole. The vacuoles appear to be stretched somewhat as if they were beginning to move centripetally. Elements of plastidome, when present, are always forced centrifugally.

Other cells taken farther up the root-tip also show the striking displacement of the osmiophilic platelets to the centripetal pole, figs. 2, 10, and 11, Plates 15 and 16. On the other hand, the plastidome elements, as shown in figs. 2 and 11, are collected at the opposite or centrifugal pole of the cells. The effect on the vacuoles in these cells is not clearly shown. Figs. 9, 14, and 15, Plates 16 and 17, are of cells taken from the peripheral layers of the root-tip a short distance above the root-cap. These cells are quite long and when cut in longitudinal section demonstrate beautifully the displacement of the osmiophilic platelets to the lighter or centripetal pole of the cells. They may be forced completely against the cell membrane, fig. 9, or may take up a position between the nucleus and the vacuome in about the centripetal one-third of the cell, figs. 14 and 15. Obviously, the vacuoles in figs. 14 and 15 are the lightest material in these cells. Never have we found the osmiophilic platelets forced inside the vacuome. Neither the pseudo-chondriome nor the plastidome is clearly shown in figs. 9, 14, and 15. However, in fig. 13, Plate 17, is a clear illustration of the striking difference in specific gravity between the osmiophilic platelets, which are as usual collected at the centripetal pole on the one hand, and the elements of the plastidome, which are displaced to the opposite or centrifugal end of the cells on the other. The middle portion of the cell which contains the nucleus is free of both osmiophilic platelets and plastidome.

3—*The Plastidome (Proplastids) and Starch Grains*

The term plastidome has been used by Bowen and others to include many structures which were formerly classed as plastids. The young plastids have been more specifically termed the proplastids and are, in general, elongated filaments or rod-shaped, in many ways resembling the filamentous mitochondria of animal cells. These bodies when impregnated with osmic acid become brownish in colour, fig. 11, Plate 16, and in older stages they often round up into archioplasts and impregnate a deep black, fig. 13, Plate 17. The proplastids stain intensively following Regaud's method and in the normal uncentrifuged cells are evenly distributed throughout the cytoplasm.

Upon ultracentrifuging the cells, the plastidome elements (filamentous proplastids) are immediately displaced to the centrifugal pole without exception, figs. 3, 11, and 12, Plates 15 and 16. In some of these cells, figs. 3 and 12, the granular pseudo-chondriosomes are also seen stratified in the same layer as the plastidome. This can only be interpreted to mean that the two structures are of the same relative specific gravity. Whether or not they represent two forms of the same material is an important question. It is therefore evident that the plastidome elements (filamentous proplastids) are sharply separated from the osmiophilic platelets on the basis of specific gravity.

In other cells where the plastids have rounded up, as in figs. 2, 5, and 13, Plates 15 and 17, they may or may not impregnate with osmic acid. However, they are always displaced to the centrifugal pole in ultracentrifuged cells. In fig. 18, Plate 17, the plastidome can be faintly seen as clear unstained bodies filling the centrifugal end of the cells. The pseudo-chondriome in these cells is forced to take up a position in the centripetal end because of the extreme density and number of plastids present. The osmiophilic platelets are not demonstrated in these cells. In still other types of cells where the starch grains are numerous and highly developed, a striking effect is noted upon ultracentrifuging, figs. 6, 21, and 23, Plates 15 and 18. The starch grains which are the densest structures in the cells are accordingly forced to the centrifugal pole where they fill roughly one-third of the cell. They often possess deeply staining rims which presumably are the remains of the plastidome elements from which they have been derived. In the middle portion of the cell, fig. 6, Plate 15, is noted a number of typical plastidome elements before and during the deposition of starch within them. A very close topographical relationship is thus observed between the plastidome and developing starch grains. Furthermore, even in

these cells a stratification of the materials is noted. The starch grains occupy the centrifugal end of the cell, the proplastids are collected into the middle portion, pseudo-chondriome and nucleus are next in order, and then a clear area of cytoplasm at the centripetal end which in many cells shows the negative image of the osmiophilic portion of the osmiophilic platelets.

In general the plastidome reacts to centrifuging as do the chloroplasts described by Mottier (1899) and Andrews (1915). Starch granules have also been shown to be comparatively heavy (Dehnecke, 1880; Heilbronn, 1914; Kostoff, 1930).

4—*The Pseudo-chondriome*

The pseudo-chondriomes, in the sense used by Bowen, consist of numerous granules scattered throughout the cytoplasm of plant cells. They are clearly demonstrated by Regaud's method and often are shown side by side with the plastidome. As pointed out by Bowen, they are readily distinguished from the osmiophilic platelets by their spherical rather than plate-like form. Then, too, they are seldom impregnated by the osmic acid methods.

When ultracentrifuged the pseudo-chondriomes are usually forced to the centrifugal pole of the cells, figs. 16 and 17, Plate 17. They frequently are found, figs. 3 and 12, Plates 15 and 16, to be stratified on the same level as the plastidome elements. Hence, as has been pointed out above, they cannot differ greatly in specific gravity from the proplastids. However, in other cells, fig. 4, Plate 15, the pseudo-chondriomes take a postion just centripetal to the plastids at the centrifugal end of the cell. In still others, fig. 18, Plate 17, the plastids are so numerous that after centrifuging they occupy the whole of the centrifugal end of the cells where they appear as clear unstained spherical bodies. The pseudo-chondriomes, being lighter than the plastids, are forced centripetally. As described above, in older cells where the starch grains are well developed, figs. 6, 21, and 23, Plates 15 and 18, the pseudo-chondriome is again forced to take up a position with the nucleus just behind the starch grains and plastidome. Here again it is very obvious that the pseudo-chondriome is not so dense as the starch grains and plastids. Finally, in cells with enormous vacuolar systems, figs. 22 and 24, Plate 18, both the pseudo-chondriome and the plastidome are displaced into the centrifugal end with the large vacuole occupying the centripetal end.

It is evident from the above description that the pseudo-chondriome is, in general, heavier than the ground cytoplasm but lighter than the plasti-

dome and starch grains. However, the pseudo-chondriomes are distinctly of a much denser material than the osmiophilic platelets, which are always displaced centripetally.

5—*Vacuome*

While it has long been known that most plant cells contain vacuoles, only recently have these structures been considered by some as the homologue of the Golgi apparatus of animal cells. After ultracentrifuging, the vacuoles may or may not be displaced. In figs. 1, 5, and 7, Plates 15 and 17, the vacuoles seem to be considerably stretched but not greatly displaced. However, in figs. 14, 15, 19, 20, 22, and 24, Plates 17 and 18, the vacuoles are observed to be displaced to the centripetal pole of the cells where they generally fuse into one large vacuole. It is clear that where the vacuolar system is well developed and the cytoplasm greatly reduced the vacuome is, with the possible exception of the diffuse lipoid, the lightest material in the cells (compare with the results of Luyet and Ernst, 1934). The osmiophilic platelets, on the other hand, are always suspended in the cytoplasm and constitute its lightest component. There is no evidence in our experiments that the vacuolar system in plants is the homologue of the animal Golgi apparatus.

6—*Lipoid Materials*

In some of the ultracentrifuged cells at the extreme centripetal end and often flattened against the cell membrane, figs. 4, 19, and 20, Plate 15 and 17, is found a mass of fat or lipoid-like substance. This material probably represents diffuse lipoids and no doubt is the lightest material in these cells. In some cells it appears to have been forced into the large vacuole, figs. 19 and 20. However, in other cells it is obviously unrelated to the vacuolar system, fig. 4.

Discussion

We wish to point out that we are well aware of the many pitfalls that confront one in entering a new and difficult field of study. Nevertheless, there is quite obviously a need for new experimental methods to supplement the usual routine cytological procedures in the study of the cytoplasmic components and inclusions of plant cells. A review of the literature on this subject convinces one that there is as yet no general agreement regarding the presence and identity of certain cytoplasmic structures. Only very recently has a number of papers appeared on this

subject, particularly referring to the problem of whether or not there is in plant cells a category homologous to the Golgi apparatus of animal cells. Bensley (1910), Guilliermond and Mangenot (1922), Parat (1928), Guilliermond (1932), and others claim that the plant vacuome is homologous to the Golgi apparatus and Trophospongium of Holmgren in animal cells. Guilliermond (1929), particularly by use of the silver nitrate impregnation and vital neutral red staining methods, has more recently extended his observations to include a wide variety of plant cells, and finds confirmatory evidence in practically every case to support his earlier view that the neutral red staining vacuolar system is the Golgi apparatus of plants. Not only does he hold this view for plants, but he has made the statement, based mainly on the hypothesis of Parat (1928), that the classical Golgi apparatus in animal cells is an artifact formed by the fusion of neutral red staining granules under the influence of the osmic acid and silver nitrate techniques. Guilliermond's observations on plant cells have been confirmed in a general way by Bose (1927), Motte (1928), Cunha (1928, *a*, *b*), and Scott (1929).

Zirkle (1932) has called attention to the close resemblance between the reticulate tannin-filled vacuoles and the Golgi apparatus. However, he is careful to point out that a number of sharp differences exist between the two and that a definite decision on this point must await further investigation.

Rice (1928), Patten, Scott, and Gatenby (1928), Weier (1932), and Nevins (1933) have for the most part failed to substantiate Guilliermond's observations, *i.e.*, that silver and osmium methods generally impregnate the vacuome. Furthermore, Weier (1932) has pointed out that "the plant vacuoles are not comparable in the slightest degree to the Golgi zone." On the other hand, Bowen (1927, 1928, 1929), using the osmic acid methods employed by animal cytologists, demonstrated in plant cells a collection of cytoplasmic bodies which he termed osmiophilic platelets. He interpreted these bodies as "probably a new category of cytoplasmic elements, hitherto unnoticed by botanists," and suggested that they are the homologue in plant cells of the Golgi apparatus in animal cells. Patten, Scott, and Gatenby (1928) immediately repeated and confirmed both the observations and interpretations of Bowen. Very recently Nevins (1933) has also observed the osmiophilic platelets in a number of androgina and in a few androcytes. While it seemed likely to her that they were not mitochondria, no theory as to their identity was advanced.

However, Guilliermond (1928), on the other hand, concludes that the osmiophilic platelets of Bowen are simply granular mitochondria, vesicular plastids, or fatty granules and that Bowen's results are to be explained

as due to faulty technique. Bose (1929) likewise thinks of the osmiophilic platelets as representing only swollen mitochondria caused by the methods used to demonstrate them. Kiyohara (1920) claims that the osmiophilic platelets represent only young plastids.

Very recently Weier (1932, *a*, *b*) has compared the plastid to the Golgi zone of animal cells. He reaches the conclusion that the plastids are the homologue of the Golgi apparatus of animal cells, a view earlier adopted by Bowen but later discarded.

From the foregoing description it is evident that the observations recorded in this paper supply further evidence which seems to demonstrate the independence of the osmiophilic platelets of Bowen from plastidome (plastids), pseudo-chondriome (mitochondria), vacuome, or lipoid granules. This evidence, which was obtained by a new experimental method, clearly shows the osmiophilic platelets to be one of the lightest structures in the cells while the plastids and mitochondria are distinctly among the heaviest. Accordingly, we are not in agreement with Guilliermond, Bose, and Kiyohara who contend that the osmiophilic platelets represent artifacts, plastids, mitochondria, or vacuome.

We have found Bowen's theory, that the osmiophilic platelets represent the homologue of the animal Golgi apparatus, the most attractive. His hypothesis seems to be supported by the weight of the evidence which is summarized below:—

(1) The osmiophilic platelets, in many cells at least, are impregnated by the osmic acid methods (Kolatchev and Mann-Kopsch-Weigl) commonly used to demonstrate the animal Golgi apparatus.

(2) The osmiophilic platelets resemble closely both the form and structure (osmiophilic cortex and osmiophobic medulla) of certain insect Golgi bodies.

(3) Bowen claims to have shown that the function of the osmiophilic platelets in the spermatogenesis of certain plants is similar to that of the Golgi bodies in animal spermatogenesis.

(4) Neither the Golgi apparatus nor the osmiophilic platelets stain with neutral red.

(5) As has been shown in this paper, the osmiophilic platelets in the root-tip of the bean move to the centripetal pole of the cells, upon centrifuging, as does the Golgi apparatus of animal cells.

Finally, the contention of Guilliermond and others that the osmiophilic platelets represent plastids or mitochondria is shown in this paper to be untenable.

Summary

It has been demonstrated by ultracentrifuging the bean root-tip at approximately 400,000 times the force of gravity that the various cellular components can be displaced and distinctly stratified. Thus, the layers may be listed in the order of their relative specific gravity, numbering from the centrifugal to the centripetal pole as follows:—

(*a*) Starch grains (when present in the cell) often possessing a plastidome cortex.

(*b*) Plastidome (proplastids and plastids).

(*c*) Pseudo-chondriome (in some instances the proplastids and pseudo-chondriomes are not definitely separated into layers).

(*d*) Cytoplasm, more or less free of starch, plastidome, pseudo-chondriome, vacuome, lipoid granules, and osmiophilic platelets.

(*e*) Osmiophilic platelets.

(*f*) Vacuome.

(*g*) Lipoid or lipoid-like material.

In ultracentrifuged cells where the starch grains are absent and the vacuoles not well developed, the osmiophilic platelets are displaced completely against the cell membrane at the centripetal pole, while the plastidome (plastids) and pseudo-chondriome (mito-chondria) are displaced to the opposite or centrifugal pole. Thus in these cells a distinct difference in density between the osmiophilic platelets, on the one hand, and the pseudo-chondriome and plastidome, on the other, is evident. The osmiophilic platelets accordingly must be looked upon as independent structures.

The nucleus may or may not be displaced by the ultracentrifuging, depending at least to some extent on the state of the development of the vacuoles and starch grains. The nucleolus is obviously the heaviest structure in the nucleus, causing in many cases an elongation of the nucleus in the direction of the centrifugal force. In some instances it may be thrown completely out of the nucleus at the centrifugal pole.

The vacuoles are affected in various ways in different types of cells, depending apparently upon their degree of development. When quite small and isolated they are frequently not completely displaced, but often elongated in the direction of the centripetal force. However, as they become more extensively developed they are displaced and often fuse at the centripetal pole of the cell.

Resemblances between the osmiophilic platelets and the Golgi bodies of insect tissues are discussed.

References

Andrews, F. M. (1915). 'Jahrb. wiss. Bot.,' vol. **56**, p. 211.
Beams, H. W., and King, R. L. (1934, *a*). 'Anat. Rec.,' vol. **59**, p. 363.
—— (1934, *b*). 'Anat. Rec.,' vol. **59**, p. 395.
—— (1935). 'J. Comp. Neurol.', vol. **61**, p. 175.
Beams, H. W., Muliyil, J. A., and Gatenby, J. B. (1934). 'Nature,' vol. **134**, p. 810.
Beams, J. W., Weed, A. J., and Pickles, E. G. (1933). 'Science,' vol. **78**, p. 338.
Bensley, R. R. (1910). 'Biol. Bull.,' vol. **19**, p. 179.
Bose, S. R. (1927). 'Nature,' vol. **120**, p. 810.
—— (1929). 'Nature,' vol. **123**, p. 258.
Bowen, R. H. (1926, *a*). 'Science,' vol. **63**, p. 620.
—— (1926, *b*). 'Science,' vol. **64**, p. 188.
—— (1927, *a*). 'Biol. Bull.,' vol. **53**, p. 179.
—— (1927, *b*). 'Anat. Rec.,' vol. **35**, p. 309.
—— (1928). 'Z. Zellforsch.,' vol. **6**, p. 689.
—— (1929, *a*). 'Z. Zellforsch.,' vol. **9**, p. 1.
—— (1929, *b*). 'Bull. Torrey Bot. Club,' vol. **56**, p. 33.
Cunha, A. G. (1928, *a*). 'C.R. Soc. Biol. Paris,' vol. **98**, p. 1017.
—— (1928, *b*). 'C.R. Soc. Biol. Paris,' vol. **98**, p. 1594.
Dangeard, P. (1923). 'Le Botaniste,' vol. **15**, p. 1.
Dehnecke, C. (1880). 'Bot. Z.,' vol. **38**, p. 795.
Drew, A. H. (1920). 'J. Roy. Micr. Soc.,' p. 295.
Guilliermond, A. (1928). 'C.R. Soc. Biol., Paris,' vol. **98**, p. 368.
—— (1929). 'Amer. J. Bot.,' vol. **16**, p. 1.
—— (1932). 'Protoplasma,' vol. **16**, p. 291.
Guilliermond, A., and Mangenot, G. (1922). 'C.R. Acad. Sci., Paris,' vol. **174**, p. 692.
Heilbronn, A. L. (1914). 'Jahrb. wiss. Bot.,' vol. **54**, p. 357.
King, R. L., and Beams, H. W. (1934). 'Proc. Soc. exp. Biol.,' vol. **31**, p. 707.
Kiyohara, K. (1930). 'Cytologia,' vol. **1**, p. 328.
Kostoff, D. (1930). 'Protoplasma,' vol. **11**, p. 177.
Luyet, B. J., and Ernst, R. A. (1934). 'Biodynamica,' No. 2, p. 1.
Mattier, D. M. (1899). 'Ann. Bot.,' vol. **13**, p. 325.
Motte, J. (1928). 'C.R. Ass. Anat.,' 23ᵉ reunion, p. 1.
Neme, D. M. (1929). 'Protoplasma,' vol. **7**, p. 423.
Nevins, B. I. (1933). 'Cellule,' vol. **41**, p. 293.
Parat, M. (1928). 'Arch. Anat. Micr.,' vol. **24**, p. 73.
Patten, R., Scott, M., and Gatenby, J. B. (1928). 'Quart. J. Micr. Soc.,' vol. **72**, p. 397.
Rice, B. B. (1928). 'Science,' vol. **56**, p. 561.
Scott, F. M. (1929). 'Amer. J. Bot.,' vol. **16**, p. 598.
Weier, T. E. (1932, *a*). 'Biol. Bull.,' vol. **62**, p. 126
—— (1932, *b*). 'Amer. J. Bot.,' vol. **19**, p. 659.
Zirkle, C. (1932). 'Z. Zellforsch.,' vol. **16**, p. 26.

Explanation of Plates

All figures are of ultracentrifuged bean root-tip cells. The direction of the centrifugal force is toward the bottom of the page. The drawings for Plate 15 were made by Mrs. Esther H. Powers. They were outlined as far as possible by the aid of a camera lucida and subsequently filled in free-hand. Plates 16, 17, and 18 are photomicrographs.

Plate 15

FIG. 1—Cells of the periblem showing osmiophilic platelets displaced to the centripetal pole. Kolatchev technique. × 2000.

FIG. 2—Cross-section of a cell of periblem farther up the root illustrating the displacement of the osmiophilic platelets to the centripetal pole and the plastidome (plastids) to the centrifugal pole. Kolatchev technique. × 2000.

FIG. 3—Cell from root-cap illustrating the displacement of the plastidome (filamentous proplastids) and the pseudo-chondriome (granular mitochondria) collected in the centrifugal end. Regaud technique. × 2000.

FIG. 4—Similar type of cell as in fig. 3. Large leucoplasts and pseudo-chondriomes are seen layered at the centrifugal pole and a fat-like mass at the centripetal pole. Champy fixation-hematoxylin stain. × 2000.

FIG. 5—Periblem cell with plastids collected at centrifugal end and poorly impregnated osmiophilic platelets are seen at the centripetal end. Nucleolus is shown forced to the centrifugal pole of the nucleus. Kolatchev technique. × 2000.

FIG. 6—Cell showing starch grains, plastidome and pseudo-chondriome. Regaud technique. × 2000.

Plate 16

FIG. 7—Low-power view showing section of periblem as in fig. 1. Osmiophilic platelets displaced to centripetal pole. Technique as in fig. 1. × 650.

FIG. 8—Enlarged view of cell as in fig. 7. Same technique. × 2000.

FIG. 9—Dermatogen cells showing displaced osmiophilic platelets. Kolatchev technique. × 2000.

FIG. 10—Periblem cell as in fig. 2. × 2000.

FIG. 11—Periblem cell showing displaced osmiophilic platelets to the centripetal pole and displaced plastidome (proplastids) at the centrifugal pole. Kolatchev technique. × 2000.

FIG. 12—Cell from root-cap illustrating the displacement of the pseudo-chondriome and mitochondria to centrifugal pole. Regaud technique. × 2000.

Plate 17

FIG. 13—Plerome cells with osmiophilic platelets as usual displaced to centripetal pole and the plastidome (leucoplasts) at the centrifugal pole and impregnated a deep black. Kolatchev technique. × 2000.

FIGS. 14 and 15—Cells from dermatogen region of tip showing displaced osmiophilic platelets and vacuoles at the centripetal pole. Kolatchev technique. × 2000.

FIGS. 16 and 17—Cells from outer plerome showing displaced nuclei and pseudo-chondriome at centrifugal pole of the cells. Regaud technique. × 2000.

FIG. 18—Similar types of cells with greatly developed leucoplasts. The unstained leucoplasts occupy the centrifugal half of cell and the pseudo-chondriome is forced into centripetal half of cell. Regaud technique. × 2000.

FIGS. 19 and 20—Root-cap cells showing the position taken by the nuclei, pseudo-chondriome, vacuoles and lipoid material. Regaud technique. × 2000.

PLATE 18

FIG. 21—Section showing a number of cells with well-developed starch grains and plastidome as in fig. 6. Regaud technique. × 650.

FIG. 22—Older plerome cells with well-developed vacuome. Plastidome and pseudo-chondriome forced to centrifugal pole of cells. Nuclei greatly stretched and displaced. Regaud technique. × 650.

FIG. 23—Enlarged photographs of cell as in figs. 6 and 21. Regaud technique. × 2000.

FIG. 24—Enlarged view of cell as in fig. 22. The nucleus is greatly stretched with the nucleolus at centrifugal pole. Regaud technique. × 2000.

576.809.514

The Action of X-Rays on Certain Bacteria

By A. T. PUGSLEY, B.Agr.Sc. (School of Agriculture); T. H. ODDIE, M.Sc.; and C. E. EDDY, D.Sc., F.Inst.P. (Natural Philosophy Laboratory, University of Melbourne)

(*Communicated by T. H. Laby, F.R.S.—Received March* 4, 1935)

1—INTRODUCTION

The action of radiation (including X-rays) on living organisms is as yet very incompletely known. Since the resulting effect is due to a number of factors, it is imperative to study first the simplest type of organism, a single cell, and to attempt to discover the variations due to different factors. In this paper an account is given of a number of experiments on *B. coli*, and a few on *Sarcina lutea* and *Phytomonas medicaginis* var. *phaseolicola*; *B. coli* was chosen because it is of simple structure, is free from clumping, and has been studied extensively in other directions.

Although much has been written on this and allied subjects, there are available few quantitative results which are essential to a solution of the problem. Throughout the literature occur many inconsistencies; some early workers considered that X-rays had no effect on bacteria,

while some found stimulation and some killing. In many instances little or no experimental evidence is brought forward in support of stated views.

Negative results with X-rays and bacteria have been obtained by a number of workers (Ref. Group 1) due, possibly, to one or more of the following causes :—

(i) doses were too small;

(ii) too many bacteria were used;

(iii) the number of surviving organisms was not determined; and

(iv) sometimes tests were made by inoculating animals with the irradiated bacteria, in which case if even a few remained undamaged by the X-rays the conclusion would be drawn that the bacteria were not killed.

Workers (Wolfenden and Forbes-Ross (1900), Gutzeit and others (1924)) who found stimulation with small doses do not seem to have counted the number of bacteria surviving after irradiation, but have based their conclusions on other effects (motility, acid production, etc.); it might be advisable to limit the term "stimulation" to an abnormal increase in the number of bacteria, but there is no experimental evidence that this occurs.

There is, however, a definite killing of bacteria (Rieder (1902), Rudis-Jicinsky (1901), Halberstaedter and Meyer (1922), Trillat (1926)) subjected to sufficient irradiation with X-rays of long wave-length.

The sensitivity varies for different bacteria; for instance, Sulkowitch (1929) found that *B. coli* is more easily killed than *Sarcina lutea*; which result agrees with the present work.

Of the quantitative work published (Ref. Group 2) the most thorough is that of Wyckoff (1930), who used *B. coli* and *B. aertryke*; he exposed freshly inoculated agar plates to the X-rays, finding an exponential relation $N/N_0 = e^{-aiT}$ between the fraction N/N_0 of the initial number N_0 of bacteria surviving a given dose iT, "a" being constant; a similar survivor curve has been obtained in part of the present work.

General conclusions to be drawn from the literature dealing with bacteria and X-rays are:

(i) bacteria are killed by X-rays when on agar, whether freshly sown or as colonies, and when in suspension;

(ii) for equal doses in r-units longer wave-lengths are more effective;

(iii) the presence of inorganic salts during irradiation enhances the lethal effect, probably by secondary radiation.

Study has been made of the action of X-rays on more complex multicellular organisms and on tissues (Ref. Group 3), but the change produced by the irradiation of agar alone, as found in the work to be described, has not been noticed by other workers, possibly because relatively large exposures are necessary. The effect may be chemical. That X-rays do produce chemical changes, especially oxidation and reduction, was shown by Chamberlain (1925) and by Roffo and Correa (1925). Risse (1929) has found that X-rays produce and also decompose hydrogen peroxide in pure water.

Better agreement is evident between results for ultra-violet light (Ref. Group 4) than between those for X-rays. Tests for an effect of ultra-violet light on agar itself appear to have yielded negative results, but Bedford (1927), using large exposures, found that bacteria are killed when sown on agar previously dosed. With a test which was not specific he detected small quantities of hydrogen peroxide on the exposed areas.

γ-rays and electrons also kill bacteria (Wells (1931), Bruynoghe and Mund (1925), Trillat (1927), Paulin (1925)); it is significant that the survivor-curve found by Wyckoff and Rivers (1930) for electrons is of the same form as those for X-rays and for ultra-violet light (Wyckoff, 1930, 1932). A detailed review of the literature is considered superfluous as others (Russ (1906), Klövekorn (1925), Mayer (1921), Clark (1922), Ellinger and Gruhn (1930), Beckwith, Olson, and Rose (1930)) are available.

2—Experimental Procedure

(*a*) *Method of applying X-rays*—The X-ray tube was of the hot filament, continuously evacuated, water-cooled type fitted with demountable electrodes (Eddy, 1932). In the majority of experiments, the target was of tungsten, and in the remaining few, of copper. The X-rays emerged, at an angle of emission of 110° between the directions of the cathode and X-ray beams, through a mica window 0·05 mm thick; wave-lengths as long as 2 A were therefore included in the emergent beam.

The tube was operated by a pulsating direct voltage (given by a Snook four-arm full-wave mechanical rectifier), at values varying from 10 to 40 kV R.M.S. (as read by a calibrated electrostatic kilovoltmeter), and with currents of from 5 to 20 milliamperes. Peak voltages corresponding to certain R.M.S. voltages were determined by sphere gap measurements.

The doses of X-rays delivered were determined by a thimble type dosimeter, and are given in *r*-units as measured by that instrument. With X-rays generated at low voltages, the wall effect produced in thimble ionization chambers is considerable, and for this reason the readings

given are not necessarily true international *r*. For experiments in which the same voltage was used, however, the wall effect would be constant, and as only relative values of the doses were required, this method of measurement was satisfactory. At the usual working voltage of 39·3 kV peak, it was found that 1 milliampere-minute equalled 513 *r*-units at the position occupied by the specimen being irradiated. Specimens to be irradiated were placed at a distance of about 2 cm from the window or 4·3 cm from the focal spot; in this way very intense X-ray beams were incident on the exposed materials.

Doses were corrected for small departures of one or two millimetres of the position of the specimens from that stated above, using the inverse square law.

(*b*) *Treatment of the Bacteria*—For the preparation of colonies a suspension of the organisms was flooded over the surface of ordinary nutrient agar in a Petri dish and the excess was poured off the plate, which was allowed to drain for a few minutes. Plates were incubated at 25° C for 24 hours in the case of *B. coli* and for 48 hours for those of *Sarcina lutea* and *Phytomonas medicaginis* var. *phaseolicola*, after which periods the colonies were of a convenient size—2 to 3 mm diameter.

The suspension used was such as to give 10 to 20 colonies per plate, making it possible to treat individual colonies.

For the sowing of plates used in experiments on agar and on freshly inoculated agar, a more concentrated suspension of bacteria was necessary, giving between 1000 and 2000 bacteria per square inch, and the plates required draining for at least 30 minutes.

The reaction of the agar was adjusted to $p_H = 7 \cdot 4$ and the control experiment was performed to ensure that this reaction was not changed by the effect of X-rays on the agar itself, the experiment being made on nutrient agar of the same kind as that used in the observations on bacteria. The agar was exposed to X-rays for such a time and in such a manner that the " dose " exceeded the maximum dose used in any of the experiments on *B. coli* in agar (10,000 *r* units). The indicators, Brom Thymol Blue and Phenol Red, were present in separate pieces of the agar during this exposure. No colour change of either indicator took place.

(*c*) *Method of Exposing and Counting Bacteria.*—The prepared Petri dishes were invariably set up with their open surfaces normal to the X-ray beam at a distance of about 2 cm from the X-ray tube window; at this distance an exposed area of about 2 sq cm was obtained. When colonies were treated they were situated in the centre of this area, the number surviving after irradiation being found by subsequent dilution

and plating. Individual colonies of approximately equal size were exposed, after which each colony with a small portion of surrounding agar was lifted from the plate with a sterile glass cover-slip and transferred to 10 cc of sterile water. The colonies were completely broken up, suitably diluted, incubated, and counted. In experiments on agar, the agar was exposed, then inoculated and incubated; but freshly sown plates, after inoculation, were first drained and allowed to dry partially for 30 minutes, and then exposed and incubated; in these experiments the number of resulting colonies per unit area was obtained from counts on four portions of the dosed area and on six adjacent control areas.

3—Results for the Effect of X-Rays on Agar

Recently Proks (1933) found that the irradiation with ultra-violet light of bacterial nutritive media made them less suitable for growth; and since in later experiments bacteria were irradiated on agar, an investigation was required first as to the action of X-rays on agar. Preliminary experiments showed that, for equal doses, the number of bacteria killed on the irradiated agar decreases greatly when the time between irradiation and inoculation is increased. Mean values from a series of ten experiments indicated that the fraction killed decreases exponentially with time; thus $n/\mathrm{N}_0 = \mathrm{K}e^{-kt}$ where K and k are constants, n/N_0 is the ratio

$$\frac{\text{number of bacteria killed per unit area}}{\text{number of bacteria originally living per unit area}},$$

and t is the time from the middle of the dose to inoculation. k was found to be approximately 1×10^{4}.

Results of a large number of experiments in which the dose was varied and t kept comparatively small were corrected for "decay," using this value of k, and mean points found showed that, for constant t, n/N_0 was very nearly proportional to the dose.

Finally, to obtain a better value of k, all experiments were taken into account, measured values of n/N_0 being corrected to a dose of 200 milliamp-minutes (103,000 r), assuming that $n/\mathrm{N}_0 = \mathrm{K}i\mathrm{T}$, where $i\mathrm{T}$ is the dose; thus n/N_0 corrected $= 200/i\mathrm{T} \times n/\mathrm{N}_0$ measured.

When t is large n is small, and the numbers of colonies appearing on the dosed and control areas are nearly equal. Errors in counting due to variations in the distribution of bacteria across the agar surface made small negative values of n/N_0 appear in four cases; these values were retained, but should not be interpreted as a stimulation of growth. Results for the decay effect are given in Table I and plotted in fig. 1.

TABLE 1—AGAR: DECAY EFFECT: MEAN POINTS: VOLTAGE 39·3 KV PEAK: TEMPERATURE 18 °C

No. of readings	Mean t sec	Mean n/N_0 corrected	$\text{Log}_{10}\, n/N_0$
38	1180	$0{\cdot}74_8$	$\bar{1}{\cdot}87_4$
29	2810	$0{\cdot}59_2$	$\bar{1}{\cdot}77_2$
14	4750	$0{\cdot}41_7$	$\bar{1}{\cdot}62_0$
13	9280	$0{\cdot}30_1$	$\bar{1}{\cdot}47_9$
10	16680	$0{\cdot}15_4$	$\bar{1}{\cdot}18_8$
10	20940	$0{\cdot}10_9$	$\bar{1}{\cdot}03_7$

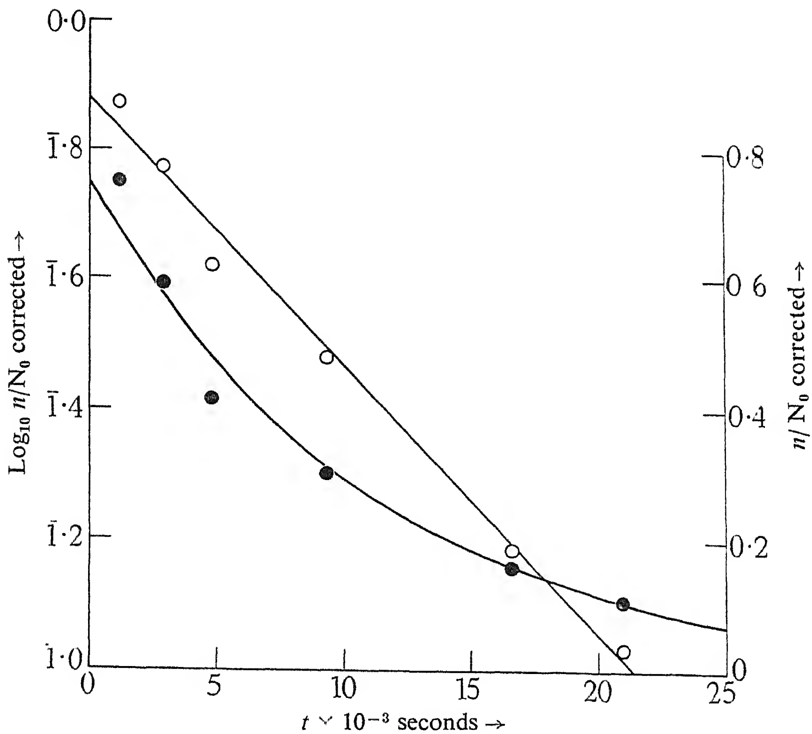


FIG. 1—Agar: Decay effect. ● n/N_0; ○ $\log_{10} n/N_0$

Using the final value $k = 9{\cdot}44 \times 10^{-5}$, readings were again corrected for " decay " by multiplying each dose by a factor giving a quantity $D = iT\, e^{-9{\cdot}44\times10^{-5}t}$.

Mean values of a number of readings are given in Table II and plotted in fig. 2, which shows that a linear relation exists

$$n/N_0 = 3{\cdot}85 \times 10^{-3}\, D.$$

TABLE II—AGAR: DOSE EFFECT: MEAN POINTS: VOLTAGE 39·3 KV PEAK: TEMPERATURE 18° C

No. of readings	Mean D m.a.-mins	Mean n/N_0
25	46	$0{\cdot}23_2$
16	96	$0{\cdot}43_7$
16	134	$0{\cdot}50_7$
16	159	$0{\cdot}66_0$
17	191	$0{\cdot}68_0$
16	225	$0{\cdot}84_6$

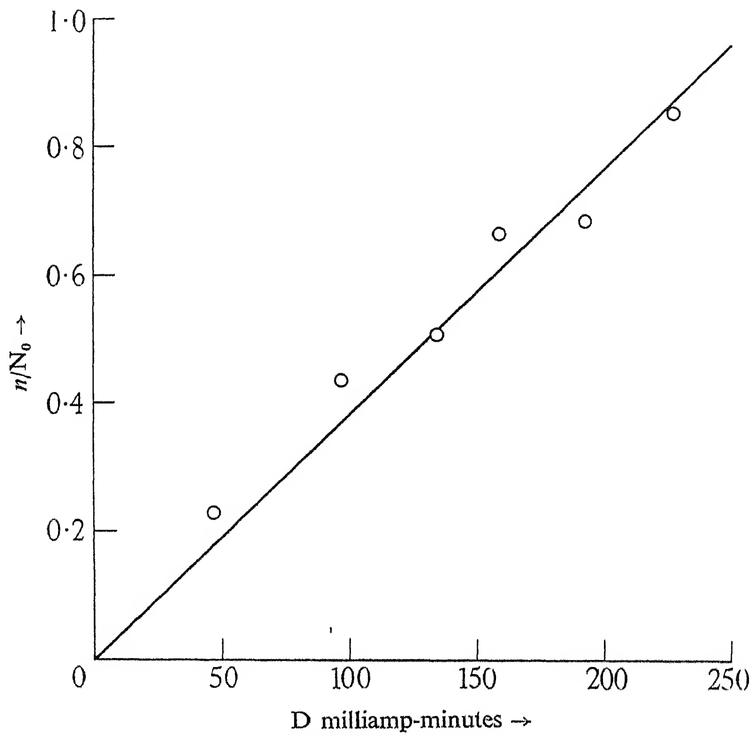


FIG. 2—Agar: Dose effect. ○ $\log_{10} n/N_0$

The value $3{\cdot}85 \times 10^{-3}$ agrees to within 3% with that obtained from the time-decay experiments, $3{\cdot}74 \times 10^{-3}$. Thus finally, using the mean, the number of bacteria killed is proportional to the dose, but fades away exponentially with time, or

$$n/N_0 = 3{\cdot}80 \times 10^{-3}\, iTe^{-0{\cdot}44\times10^{-3}t}, \tag{1}$$

where iT is in milliamp.-minutes (1 m.a. min = 513 r) and t is in seconds.

This effect of the X-rays on the agar introduces into experiments with bacteria on agar an error which is barely appreciable (about 2%). It may be explained assuming that X-rays absorbed at the surface of the agar produce a toxic poison which decomposes in a unimolecular manner.

Throughout this paper let

t = time in seconds,
T = duration of dose in minutes or seconds as specified,
i = tube current in milliamps,
x = number of X-ray quanta crossing the plane of bacteria or agar at right angles to the X-ray beam, per second per unit area,
K, k, etc., = constants.
N_0 = number of bacteria per unit area originally living,
N = number of bacteria per unit area living after irradiation, and
n = number of bacteria per unit area killed.

A certain fraction of the X-quanta will be absorbed by the agar at the surface layer; these quanta will vary in energy content with wave-length, but for the quality of radiation considered let one quantum, on the average, produce q molecules of toxic poison. Then the number of molecules of poison produced per unit area in time dt is

$$k_{11}\, qx\, dt. \tag{2}$$

If C = the number of molecules of poison per unit area, we have in time dt, increase in C due to absorbed quanta

$$(dC)_1 = k_{11}\, qx\, dt \tag{3}$$

using (2),

and the number of molecules decomposing is proportional to C, *i.e.*,

$$(dC)_2 = -\, k_{12}\, C\, dt. \tag{4}$$

Thus

$$dC/dt = \frac{(dC)_1 + (dC)_2}{dt} = k_{11}\, q\, x - k_{12}\, C. \tag{5}$$

Integrating, with initial conditions C = O when t = O, the concentration at the end of the dose (time T) is

$$C_T = (k_{11}\, qx/k_{12})\,(1 - e^{-k_{12}T}). \tag{6}$$

Then if τ is the time from the end of the dose to inoculation of the agar for decomposition alone we have

$$dC/dt = -\, k_{12}C \tag{7}$$

using (4).

Whence

$$C_\tau = C_T \, e^{-k_{12}\tau} \tag{8}$$

and using (1·42),

$$C_\tau = (k_{11}\, qx/k_{12})\,(1 - e^{-k_{12}T})\, e^{-k_{12}\tau}. \tag{9}$$

When the rate of decomposition is slow, as in practice, $k_{12}T$ is small and (9) approximates to

$$C_{t'} = C_\tau = k_{13} x T \, e^{-k_{12}t'} \tag{10}$$

where

$$k_{13} = k_{11}\, q \tag{11}$$

and

$$t' = T/2 + \tau = \text{time between the middle of dose and inoculation.} \tag{12}$$

(The approximation used is $1 - e^{-k_{12}T}$

$$= 1 - 1 + k_{12}T - k^2_{12}\, T^2/|\underline{2} + k^3_{12}\, T^3/|\underline{3} - \ldots$$
$$= k_{12}T\,(1 - k_{12}T/|\underline{2} + k^2_{12}\, T^2/|\underline{3} - \ldots)$$
$$= k_{12}T\, e^{-\frac{1}{2}k_{12}T}).$$

Now for each bacterium sown on the dosed area let the chance of reaction with sufficient molecules of poison to cause death be proportional to the r-th power of the concentration; that is, r molecules of poison are required to kill one bacterium.

Then the number killed is

$$n = k_{14}\, N_0\, C^r_{t'}, \tag{13}$$

$$= k_{14}\, N_0\, k^r_{13}\, (xT)^r\, e^{rk_{12}t'} \tag{14}$$

using (10).

But the number of quanta per second is proportional to the tube current, *i.e.*,

$$x = k_1\, i \tag{15}$$

and so finally

$$n/N_0 = K_1\, (iT)^r\, e^{-K_2 t} \tag{16}$$

where

$$K_1 = k_{14}\, k^r_{13}\, k^r_1, \tag{17}$$

and

$$K_2 = r\, k_{12}. \tag{18}$$

When $r = 1$, (16) becomes

$$n/N_0 = K_1\, iT\, e^{-K_2 t}, \tag{19}$$

which fits the experimental results recorded in Tables I and II. Values of r greater than unity also give agreement.

It is possible that the X-rays might accelerate the decomposition of the poison molecules, but for agreement with experiment it is necessary to assume that this effect is negligible.

4—Experiments on Agar Freshly Sown with *B. coli* at Constant Voltage

Over 200 exposures to X radiation were made with agar freshly sown with *B. coli*, with a range of doses from 0 to 13 millampere-minutes (0 to 667 *r*). The readings were found to be scattered about a straight line when the logarithm of the fraction of bacteria surviving a given dose ($\log N/N_0$) was plotted against dose (iT), and it was possible therefore to use mean points for a series of ranges of dosage.

The logarithmic method of plotting readings has been adopted uniformly throughout, and wherever survivor-curves are mentioned this form is assumed. Mean points are tabulated in Table III and plotted in fig. 3, discussion being deferred to a later section of the paper.

5—Experiments on Colonies of *B. coli* at Constant Voltage

It was considered of interest to discover any differences which might exist between the actions of X rays on bacteria freshly sown, *i.e.*, separated from each other, and in colonies. The latter were 24 hours old in all experiments except in an age test dealt with later. Individual readings on different days showed poor agreement, but points obtained in a single experiment, with colonies on the same plate, usually lay on a fairly smooth curve. Eighteen out of twenty-two survivor-curves were concave upwards, the remaining four being approximately straight lines; which is considered conclusive evidence that the curvature was not due to random variation of readings. The mean points (Table IV) give the curve shown in fig. 4.

6—Various other Factors Tested

(*a*) Experiments described in §5 above were so arranged that the orders of dosing and of dilution of the colonies were varied; the quantity $\log_{10} \frac{N}{N_0}/iT$ was calculated for each reading and means found for readings with longer and shorter times between exposure and dilution of the colony. Values obtained were

(i) for time specified 20 minutes, mean $\log_{10} N/N_0 = -0{\cdot}194$.
(ii) for time specified 45 minutes, mean $\log_{10} N/N_0 = -0{\cdot}196$.

TABLE III—*B. coli*: INOCULATED PLATES: MEAN POINTS: VOLTAGE 39·3 KV PEAK: TEMPERATURE 18° C

No. of readings	Mean iT m.a.-mins	Mean $\log_{10} N/N_0$
52	2·35	$-0·47_8$
40	4·73	$-0·84_5$
33	6·90	$-1·30_6$
47	9·05	$-1·75_6$
35	11·28	$-1·90_3$

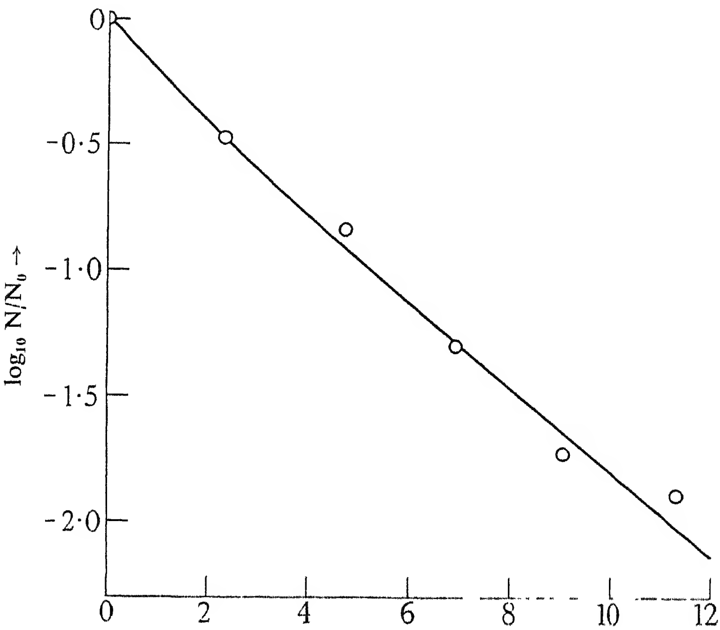


FIG. 3—Agar freshly sown with *B. coli*

No significant difference is evident and there appears to be no delayed action on the organisms.

(*b*) A change in the composition of the medium on which dosed colonies were grown produced no appreciable change in the killing rate. For this test a medium was used differing from ordinary nutrient agar and composed of

distilled water	1000 cc
acid potassium phosphate	1 gm
magnesium sulphate	0·2 gm
ammonium phosphate	10 gm
glycerin	45 gm
agar-agar	20 gm

TABLE IV—*B. coli*: 24-HOUR COLONIES: MEAN POINTS: VOLTAGE 39·3 KV PEAK: TEMPERATURE 18° C

No. of points	Mean iT m.a.-mins	Mean $\log_{10} N/N_0$
19	7·37	−1·68
21	13·84	−2·78
22	21·06	−3·31
18	28·52	−4·21
17	38·29	−5·28

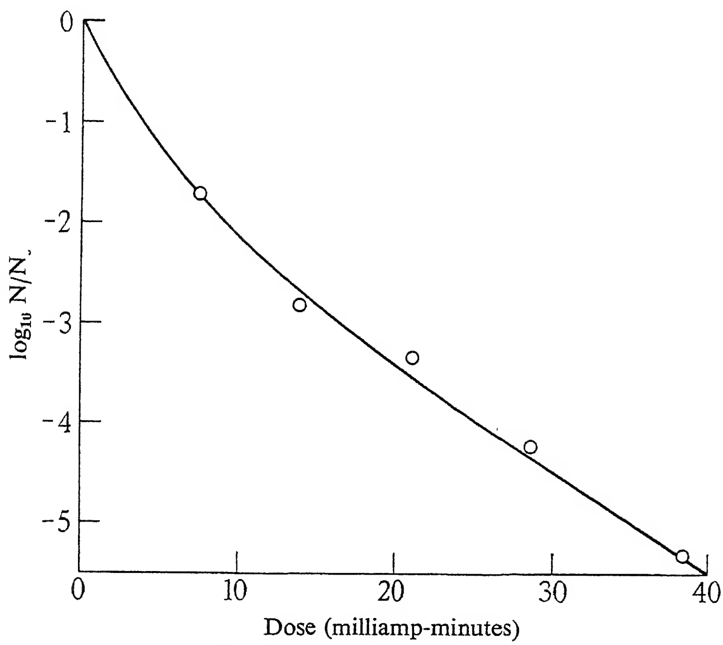


FIG. 4—24-hour colonies of *B. coli*

(*c*) Evidence was obtained that a considerable portion of the lethal action of the X-rays used was due to the longer wave-lengths which were appreciably absorbed in a few millimetres of the agar. *Sarcina lutea* was sown at a depth in the agar as well as on the surface. Doses of 100 and 200 milliampere-minutes were given, and it was found that whereas a complete kill was effected at the surface for each dose, the fraction killed at a depth of about 5 mm was quite small.

(*d*) An order of sensitivity increasing from *Sarcina lutea* through *B. coli* to *Phytomonas medicaginis* var. *phaseolicola* was found, this order being the same for each type of experiment—single bacteria, colonies, and dosed agar.

(*e*) Four experiments with colonies of different ages (one, three, six, and nine days old) showed that the older colonies were slightly more easily killed. This might result from the fact that with older colonies the living bacteria are situated round the edges as a thin layer; the X-rays, therefore, are not subjected to any appreciable absorption before reaching the lower surface of the colony.

TABLE V—*B. coli*: 24-HOUR COLONIES: EFFECT OF VOLTAGE ON KILLING RATE: TEMPERATURE 18° C

I R.M.S. voltage	II Peak voltage kV	III Relative killing rate	IV Product of I and III
10	14·1	2·48	25
20	28·3	1·48	30
27·8	39·3	1·00	28
30	42·5	0·95	29
40	56·6	0·69	28

(*f*) Some workers have reported an increase in the killing rate with increase in the wave-length of the X-rays used. From the survivor-curves obtained for voltages between 16 and 55 kV peak it was possible to investigate the variation of bactericidal effect with voltage (*i.e.*, with wave-length). Over this voltage range the general shape of the survivor-curves remained the same. Results for *B. coli* are given in Table V. The killing rate given is the slope of the logarithmic survivor-curve for a constant dose expressed in *r*-units. It would appear that the killing rate is approximately inversely proportional to the X-ray tube voltage, *i.e.*, that longer wave-lengths are more effective for killing purposes. It may be possible, however, to explain this apparent increase in killing rate with wave-length as due to errors introduced by the method of measuring dosage. For longer wave-lengths the fraction of an X-ray beam absorbed in air is not necessarily a measure of the fraction of the same beam which is absorbed in the bacteria colony. (The values of the absorption coefficients for air and water indicate that a greater dose is delivered in water for a constant dose in air as the wave-length is increased.)

7—THEORETICAL

The mode of death of bacteria under the influence of radiations may be explained in several ways. One group of writers suggests that the action is one of chance, that is, a bacterium has a definite chance of being hit and killed by a single quantum or electron, or by two or more con-

secutive quanta. This explanation was first put forward by Crowther (1924) to account for the results of Strangeways and Oakley (1923–24); it has been amplified by Condon and Terrill (1927), Holweck (1928–29), Glocker (1932), Dessauer (1933), and Dänzer, (1934).

Falk and Winslow (1926) have discussed the killing of bacteria by a toxic poison; they favour a mechanism analogous with the " mass action " of chemical change.

Gates (1931) and Brooks (1918–19) incline to the " resistance " theory, which assumes that there is a distribution of resistance to poison (or to X-rays, etc.) among the bacteria. Rahn (1929–30) suggests that the main basis of the killing effect is chance, and that deviations from a strictly exponential kill are due to variations of resistance.

Several possible mechanisms are dealt with below.

(*a*) *Simple Quantum-Hit Explanation*—Assume that if p quanta are absorbed in, or p electrons strike, a certain vulnerable volume of a bacterium, the latter dies. Let " a " be the area of cross-section of this sensitive volume exposed to the radiation (it being immaterial whether the quantum and the bacterium each have effective areas as these may be combined). Then it has been shown Holweck (1928-29), Glocker (1932) that

$$N/N_0 = (1 + axt + (axt)^2/\underline{|2} + (axt)^3/\underline{|3} \ldots + (axt)^{p-1}/\underline{|p-1})e^{-axt} \quad (20)$$

where x and t are as in § 3, and N/N_0 is the fraction of bacteria surviving the given dose. When $p = 1$ (20) becomes

$$N/N_0 = e^{-axt}, \quad (21)$$

which form fits the results for freshly inoculated plates given in § 4. Theoretical curves with values of $p > 1$, when plotting as usual $\log N/N_0$ against dose, are all concave downwards.

The vulnerable volume of a bacterium might vary for the second and later hits, and it has been considered advisable to solve the equations for typical possible cases.

Let a_n = area for the nth hit.

Then, for two hits to kill

$$N/N_0 = \frac{1}{a_1 - a_2}(a_1 e^{-a_2xt} - a_2 e^{-a_1xt}), \quad (22)$$

and in the particular case $a_1 = 2a_2 = 2a$, this becomes

$$N/N_0 = 2e^{-axt} - e^{-2axt} \quad (23)$$

For three hits, particular case $a_1 = 3a$, $a_2 = 2a$, $a_3 = a$,

$$N/N_0 = 3e^{-axt} - 3e^{-2axt} + e^{-3axt}. \tag{24}$$

Again on the assumption that a bacterium may be killed in either of two ways:

(1) by one quantum hit on an area a_1, or

(2) by two quantum hits on a less vulnerable area a_2,

leads to the relation

$$N/N_0 = (1 + a_2xt)\, e^{-(a_1+a_2)xt}. \tag{25}$$

Similarly, the assumption that certain fractions of a number of bacteria hit once, twice, and so on, die, leads to a curve having similar properties to the above.

All the above curves, except (21), possess negative curvature when plotted semi-logarithmically. Thus, in general, if one quantum hit is required for a kill, a straight line survivor-curve results, while multiple-hit curves all have negative curvature (*i.e.*, are concave downwards).

(*b*) *Effect of Clumping of Organisms on Shape of Survivor-Curve*—Clumping together of bacteria when being diluted and plated out for counting purposes may introduce a serious error. If there are N_0 bacteria present let

p_1N_0 be single,

p_2N_0 be in clumps of two,

. . . .

$p_s N_0$ be in clumps of s.

And let n bacteria be killed, so that the number living

$$N = N_0 - n. \tag{26}$$

All having an equal chance of being killed, in the rth group in general, p_rn will be killed.

Any one clump containing one or more living bacteria would produce a colony and would be counted as one living bacterium, and the chance of all members of a clump of r being killed is approximately

$$\left(\frac{p_rn}{p_rN_0}\right)^r = \left(\frac{n}{N_0}\right)^r. \tag{27}$$

And there are $p_r N_0/r$ clumps of r, so that the number of r clumps in which all are killed is

$$\frac{p_r N_0}{r}\left(\frac{n}{N_0}\right)^r. \tag{28}$$

Hence the measured number dead (all groups) will be

$$n' = (n/N_0) \times N_0 p_1/1 + (n/N_0)^2 \times N_0 p_2/2 + \ldots + (n/N_0)^s \times N_0 p_s/s. \tag{29}$$

Whence

$$n'/N_0 = (n/N_0)\, p_1/1 + (n/N_0)^2\, p_2/2 + \ldots + (n/N_0)^s\, p_s/s \tag{30}$$

and the measured number of bacteria in the control will be

$$N'_0 = p_1 N_0 + p_2 N_0/2 + \ldots + p_s N_0/s \tag{31}$$

$$= qN_0 \text{ say,} \tag{32}$$

therefore using (30) and (32)

$$\frac{n'}{N'_0} = \frac{(n/N_0)\, p_1/1 + (n/N_0)^2\, p_2/2 + \ldots + (n/N_0)^s\, p_s/s}{q}. \tag{33}$$

The observed fraction living is

$$N'/N'_0 = 1 - n'/N'_0. \tag{34}$$

Now if values of $p_1, p_2, \ldots p_s$ are known N/N_0 may be found in terms of N'/N_0 by means of the above relations (33) and (34). Consideration will show that clumping would cause a straight line survivor-curve $\log_e N/N_0 = -\, ai\mathrm{T}$ to simulate a multiple-quantum-hit curve.

Examination of *B. coli* (diluted in the manner employed in this work) under the microscope revealed no clumping of the organisms, but *Sarcina lutea* did show clumping. Three rough counts with the latter organisms showed that about

3·5% were single,
46·9% were clumps of two,
17·7% were in clumps of three,
30·4% were in clumps of four, and
1·5% were in clumps of five.

These figures give rough values for $p_1, p_2, \ldots p_5$, leading to a correction formula

$$N'/N'_0 = 2{\cdot}46\, N/N_0 - 2{\cdot}20\, (N/N_0)^2 + 0{\cdot}97\, (N/N_0)^3 - 0{\cdot}22\, (N/N_0)^4 + 0{\cdot}007\, (N/N_0)^5. \tag{35}$$

The results of a typical experiment, at constant voltage (39·3 kV peak) on colonies of *Sarcina lutea* are plotted in fig. 5. Eight similar survivor-curves, which showed a negative curvature when uncorrected, gave approximate straight lines or curves with slight positive curvature after correction for clumping. The uncertainty of the values of constants in the correction formula (35) made it inadvisable to carry out detailed work on *Sarcina lutea*.

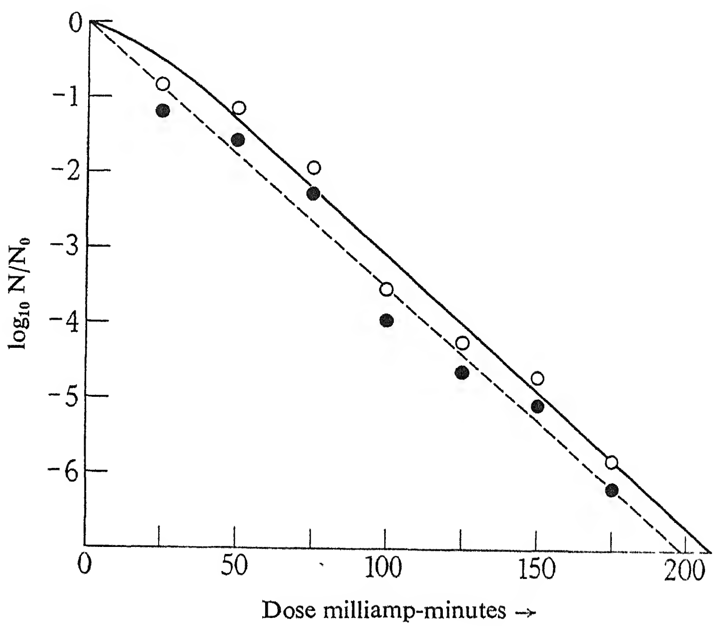


FIG. 5—Clumping effect. —o— $\log_{10} N'/N'$; - - - ● - - - $\log_{10} N/N_0$

(*c*) *Absorption of the X-rays in Top Layers of Colony*—Appreciable absorption of radiation in the upper parts of a colony would give a positive curvature to what would otherwise be a linear survivor-curve.

Let

N'_0 = original number of living bacteria per unit volume,

h = height of colony,

r = radius of base of colony, and

μ = absorption coefficient for the bactericidal radiation.

It is postulated, for simplicity (*a*) that the shape of the colony is conical, and (*b*) that the organisms are killed in a one quantum-hit-manner.

Taking an element of volume bounded by two adjacent cones of shape similar to that of the colony, and integrating, an expression is obtained

$$N = N'_0 \pi h/r \int_{z=0}^{z=r} z^2 \, e^{-axt \, e^{-\mu h(1-z/r)}} \, dz, \tag{36}$$

where N is the number of organisms per colony surviving bombardment by X-quanta per second per unit area for t seconds.

Putting $z = ry$ and using the relation

$N_0 = \frac{1}{3}\pi h r^2 N'_0 =$ number of living bacteria originally in the colony, (36) becomes

$$N/N_0 = 3 \int_0^1 (1-y)^2 \, e^{-axt \, e^{-\mu h y}} \, dy. \tag{37}$$

No simple analytical solution is available for the expression (37), and resort must be made either to convergent series for small values of the dose xt and asymptotic series for larger values or to graphical methods; the latter were more convenient. Substituting iT milliampere-minutes for xt, best values to fit the *B. coli* colony survivor-curve were found to be $a = 1{\cdot}02$ and $\mu h = 1{\cdot}85$, which with (37) give the curve in fig. 4.

(*d*) *Sensitivity Distribution—Graded or Otherwise*—If the one quantum-hit mechanism of killing is valid, but if the vulnerable volumes of bacteria of the same kind vary, the survivor-curve will have a positive curvature. For example, if half the bacteria has an effective area a_1 and half a_2 then

$$N/N_0 = \tfrac{1}{2}\left(e^{-a_1xt} + e^{-a_2xt}\right). \tag{38}$$

Alternatively, let a bacterium die when it has absorbed radiation energy equal to or greater than Q; and let $m\,d$Q bacteria require lethal energies lying between Q and Q + dQ. In order to obtain a survivor-curve $N/N_0 = e^{-axt} = e^{-bQ}$, the condition

$$m/N_0 = be^{-bQ}$$

must hold. This is the required sensitivity distribution.

8—Discussion

Having considered in § 7 various possible killing mechanisms, the most reasonable must be selected. It should be borne in mind that experiment has given survivor-curves (1) for inoculated plates a straight line, and (2) for colonies a curve with positive curvature. The former precludes all multi-quantum-hit interpretations. For results with inoculated plates there remain two possible explanations: (i) one quantum-hit

to kill, § 7 (*a*), and (ii) sensitivity distribution § 7 (*d*). The latter (ii) is not altogether satisfactory for a number of reasons:

(1) To agree with experiment, the sensitivity must vary from zero to infinity.
(2) The sensitivity distribution required (equation (38)) is not like a common probability curve.
(3) It leaves open the possibility of a bacterium being " half killed."

If, therefore, we assume mechanism (i) to be correct and turn to the colony survivor-curve, this may be interpreted in three ways, by—

(*a*) one quantum-hit to kill and absorption in the top layers of the colony,
(*b*) one quantum-hit to kill and graded sensitivity, and by
(*c*) a sensitivity distribution.

The latter (*c*) may be eliminated for reasons already stated. If (*b*) is to be accepted, and if a mixture of bacteria with two different " sensitive volumes " is assumed, a curve is obtained which fits the results for colonies

$$\mathrm{N}/\mathrm{N}_0 = 0\cdot058\, e^{-0\cdot242 i\mathrm{T}} + 0\cdot942\, e^{-0\cdot512 i\mathrm{T}}, \tag{39}$$

in which case 6% of the organisms would have a sensitivity of about half that of the remainder; which conclusion is in direct opposition to the view held by most writers that " dividing cells are more sensitive." Taking, then, mechanism (*a*) as most plausible, a comparison may be made between values of " *a* " obtained by the two methods (sown plates and colonies); but a correction must be made for variations in intensity across the X-ray beam. These variations add a correction factor to the theoretical curve $\mathrm{N}/\mathrm{N}_0 = e^{-ai\mathrm{T}}$ giving

$$\mathrm{N}/\mathrm{N}_0 = e^{-a\overline{i\mathrm{T}}}\{1 + 0\cdot0483\, a^2\, (\overline{i\mathrm{T}})^2\}, \tag{40}$$

the constant 0·0483 being obtained from relative intensity measurements at different points in a cross-section of the X-ray beam (using a photographic method in conjunction with a Moll microphotometer). $\overline{i\mathrm{T}}$ denotes the mean dose.

Inserting values in (40) to fit the results in Table III,

$$\mathrm{N}/\mathrm{N}_0 = e^{-0\cdot492\overline{i\mathrm{T}}}\{1 + 0\cdot0483\, (0\cdot492\, \overline{i\mathrm{T}})^2\}. \tag{41}$$

(This curve is drawn in fig. 3. The slight curvature shown by the experimental results is also evident in those of Wyckoff (1930).)

Thus $a = 0 \cdot 492$ for inoculated plates, and, using experimental values,

$$\text{dose at centre of beam} = 1 \cdot 60 \text{ mean dose,}$$
$$a = 0 \cdot 637 \text{ for colonies.}$$

The value for colonies is 25% higher than that for single bacteria, but it should be remembered that equation (37) was derived assuming an approximate cone shape for the colony, and neglecting the effect of scattered radiation in the colony, which would make the observed values of both "a" and "μh" too large.

These experiments were carried out partly in the School of Agriculture and partly in the Natural Philosophy Laboratory of the University of Melbourne. The writers wish to acknowledge the assistance of Professor S. M. Wadham with the bacteriological side of the paper, and of Professor T. H. Laby, F.R.S., who made available the facilities of the Natural Philosophy Laboratory. Thanks are also due to Professor W. E.. Agar, F.R.S., Dr. C. H. Kellaway, and Dr. F. M. Burnet for advice and criticism, and especially to Mr. M. H. Belz for a thorough examination of the mathematical sections. The writers are also indebted to Dr. E. H. S. Burhop for reading the proofs.

Summary

X-rays (0·25–2 A) kill *Sarcina lutea*, *B. coli*, and *Phytomonas medicaginis* var. *phaseoliocla*, the order stated being that of increasing sensitivity and the relative sensitivities being about 0·3, 1, and 3.

Irradiation impairs the nutrient agar in a manner consistent with the supposition of the production of a toxic poison.

For equal doses in *r*-units as measured, it was found that a higher killing rate was obtained with the longer wave-lengths. The possibility that this is due to the method by which doses were measured, and not to a more potent action of the longer wave-lengths, is discussed.

Bacteria irradiated singly on agar survive in an exponential manner; a correction factor being introduced for lack of uniformity of the X-ray beam.

Bacteria irradiated in colonies yield a survivor-curve differing from that for single bacteria. The shape and constants of the curve are consistent with a one-quantum-hit-to-kill mechanism, with the addition of effects due to the absorption and scattering of X-rays in the colony.

Various modes of killing being considered, the one-quantum-hit-to-kill explanation due to Crowther appears the most satisfactory.

References

Group 1

Bean (1903). 'J. Adv. Ther.,' vol. **21**, p. 362.
Beauregard and Guichard (1897). 'C. R. Soc. Biol., Paris,' vol. **4**, p. 803.
Beck and Schultz (1896). 'Z. Hyg. InfektKr.,' vol. **23**, p. 490.
Bergonie and Mongour (1897). 'Bull. Acad. Méd., Paris,' vol. **38**, p. 66.
Berton (1896). 'C.R. Acad. Sci., Paris,' vol. **123**, p. 109.
Blaise and Sambuc (1897). 'C.R. Soc. Biol. Paris,' vol. **4**, p. 689.
Blakie (1897). 'Scot. med. and surg, J.,' vol. **1**, p. 429.
Dozois, Tittsler, Lisse, and Davey (1932). 'J. Bact.,' vol. **24**, p. 123.
Levy-Dorn (1899). 'Deuts. med. Wschr.,' p. 194.
Minck (1896). 'Münch. med. Wschr.,' vol. **43**, p. 101.
Musham (1897). 'Deuts. med. Wschr.,' p. 71.
Newcomer (1917). 'J. Exp. Med.,' vol. **26**, pp. 657, 841.
Perry (1919). 'Amer. J. Roentgenol.,' vol. **6**, p. 464.
Perry and Kolmer (1918). 'Amer. J. Immun.,' vol. **3**, p. 247.
Pott (1897). 'Lancet,' vol. **11**, p. 1314.
Russ (1906). 'Arch. Hyg. Berl.,' vol. **56**, p. 341.
Sabrazès and Rivière (1897). 'C. R. Acad. Sci., Paris,' vol. **124**, p. 979.
Schiff (1898). 'Wien Arch. Derm. Syph.,' vol. **42**.
Wilson (1900). 'Clinique (Chicago),' vol. **19**, p. 613.
Wittlin (1896). 'Zbl. Bakt.,' Section 2, vol. **2**, p. 676.
Zeit (1901). 'J. Amer. Med. Ass.,' vol. **37**, p. 1432.

Group 2

Brown, Luck, Sheets, and Taylor (1933). 'J. Gen. Physiol.,' vol. **16**, p. 397.
Burger (1931). 'Bull. Basic Sci. Res.,' vol. **3**, p. 71.
Clark and Boruff (1929). 'Science,' vol. **70**, p. 74.
Claus (1933). 'J. Exp. Med.,' vol. **57**, p. 335.
Holweck (1928). 'C.R. Acad. Sci., Paris,' vol. **186**, pp. 1203, 1318.
—— (1929). *Ibid.*, vol. **188**, p. 200.
—— (1929). *Ibid.*, vol. **188**, p. 197.
Jolkevitsch (1927). 'Ann. roentgenol., Paris,' vol. **2**, p. 278.
Klövekorn (1925). 'Strahlentherapie,' vol. **20**, p. 354.
Klövekorn and Gaertner (1926). 'Strahlentherapie,' vol. **23**, p. 148.
Lacassagne (1929). 'C.R. Acad. Sci., Paris,' vol. **186**, p. 1316.
Meyer (1923). 'Klin.-therap. Wschr.,' February 12, p. 297.
Norris (1931). 'Bull. Basic Sci. Res.,' vol. **3**, p. 21.
Pauli and Sulger (1928). 'Strahlentherapie,' vol. **29**, p. 128.
Peacock and Whitby (1927). 'Brit. J. exp. Path.,' vol. **8**, p. 43.
Rice and Reed (1931). 'Canad. J. Res.,' vol. **5**, p. 122.
Schepmann and Flecke (1926). 'Klin. Wschr.,' vol. **5**, p. 1608.
Wyckoff (1930). 'J. Exp. Med.,' vol. **52**, pp. 435, 769.

Group 3

Ancel and Vintemberger (1924). 'C.R. Soc. biol., Paris,' vol. **91**, p. 1271.
Crowther (1926). 'Nature,' vol. **118**, p. 86.

Crowther (1926). 'Proc. Roy. Soc.,' B, vol. **100**, p. 390.
Ewing (1926). 'Amer. J. of Roentgenol.,' vol. **15**, p. 93.
Hance and Clark (1926). 'J. Exp. Med.,' vol. **43**, p. 61.
Klövekorn and Gaertner (1927). 'Strahlentherapie,' vol. **24**, p. 548.
Nemenow (1926). 'Brit. J. Radiol.,' vol. **31**, p. 302.
Packard (1927). 'J. Cancer Res.,' vol. **11**, p. 1.
Ponzio (1926). 'Brit. J. Radiol.,' vol. **31**, p. 502.
Strangeways and Hopwood (1926). 'Proc. Roy. Soc.,' B, vol. **100**, p. 283.
Strangeways and Oakley (1923–24). 'Proc. Roy. Soc.,' B, vol. **95**, p. 373.
Terrill (1927). 'J. Cancer Res.,' vol. **11**, p. 293.
Wood (1924). 'Amer. J. Roentgenol.,' vol. **12**, p. 474.

Group 4

Barnard and Morgan (1903). 'Proc. Roy. Soc.,' B, vol. **72**, p. 126.
Bayne-Jones and van der Lingen (1923). 'Johns Hopk. Hosp. Bull.,' vol. **34**, p. 11.
Becquerel (1910). 'C. R. Acad. Sci. Paris,' vol. **151**, p. 86.
Browning and Russ (1917–19). 'Proc. Roy. Soc.,' B, vol. **90**, p. 33.
Burge (1917). 'Amer. J. Physiol.,' vol. **43**, p. 429.
Downes and Blunt (1877). 'Proc. Roy. Soc.,' B, vol. **26**, p. 488.
Fisher and McKinley (1927). 'J. Infect. Dis.,' vol. **40**, p. 399.
Gates (1931). 'Stud. Rockefeller Inst. Med. Res.,' vol. **73**, pp. 9, 27; vol. **76**, p. 3.
Henri and Cernovodeanu (1910). 'C.R. Acad. Sci., Paris,' vol. **150**, p. 52.
Hertel (1905). 'Z. allg. Physiol.,' vol. **5**, p. 95.
Mashimo (1919). 'Kyoto Univ. Coll. Sci.,' vol. **4**, p. 1.
Thiele and Wolf (1907). 'Arch. Hyg.,' vol. **60**, p. 29.
Ward (1893). 'Proc. Roy. Soc.,' B, vol. **54**, p. 472.
Wiesner (1907). 'Arch. Hyg.,' vol. **61**, p. 1.
Wyckoff (1932). 'J. Gen. Physiol.,' vol. **15**, p. 351.

Other Citations

Beckwith, Olson, and Rose (1930). 'Proc. Soc. exp. Biol. N.Y.,' vol. **27**, p. 285.
Bedford (1927). 'Brit. J. exp. Path.,' vol. **8**, p. 437.
Brooks (1918–19). 'J. Gen. Physiol.,' vol. **1**, p. 61.
Bruynoghe and Mund (1925). 'C.R. Soc. Biol., Paris,' vol. **92**, p. 211.
Chamberlain (1925). 'Phys. Rev.,' vol. **26**, p. 525.
Clark (1922). 'Physiol. Rev.,' vol. **2**, p. 277.
Condon and Terrill (1927). 'J. Cancer Res.,' vol. **11**, p. 324.
Crowther (1924). 'Proc. Roy. Soc.,' B, vol. **96**, p. 207.
Dänzer (1934). 'Z. Phys.,' vol. **89**, p. 421.
Dessauer (1933). 'Z. Phys.,' vol. **84**, p. 218.
Eddy (1932). 'J. Sci. Instr.,' vol. **9**, p. 354.
Ellinger and Gruhn (1930). 'Strahlentherapie,' vol. **38**, p. 58.
Falk and Winslow (1926). 'J. Bact.,' vol. **11**, p. 1.
Gates (1931). 'Stud. Rockefeller Inst. Med. Res.,' vol. **73**, pp. 9, 27; vol. **76**, p. 3.
Glocker (1932). 'Z. Physik.,' vol. **77**, p. 653.
Gutzeit, Brinkmann, and Kötshcau (1924). 'Münch. med. Wschr.,' vol. **71**, p. 162.
Halberstaedter and Meyer (1922). 'Fortschr. Röntgenstr.,' vol. **29**, p. 489.

Holweck (1928). 'C.R. Acad. Sci., Paris,' vol. **186**, pp. 1203, 1316.
—— (1929). *Ibid.*, vol. **188**, p. 200.
Klövekorn (1925). 'Strahlentherapie,' vol. **20**, p. 354.
Mayer (1921). 'Amer. Rev. Tuberc.,' vol. **5**, p. 75.
Paulin (1925). 'C.R. Soc. Biol., Paris,' vol. **82**, p. 61.
Proks (1933). 'Lait.,' vol. **13**, p. 331.
Rahn (1929–30). 'J. Gen. Physiol.,' vol. **13**, pp. 179, 395.
Rajewsky and Dänzer (1934). 'Z. Physik,' vol. **89**, p. 412.
Rieder (1898). 'Münch. med. Wschr.,' vol. **45**, pp. 101, 773.
—— (1902). *Ibid.*, vol. **49**, p. 402.
Risse (1929). 'Strahlentherapie,' vol. **34**, p. 578.
Roffo and Correa (1925). 'Strahlentherapie,' vol. **19**, p. 541; vol. **20**, p. 574.
Rudis-Jicinsky (1901). 'N.Y. Med. J.,' vol. **73**, p. 364.
Russ (1906). 'Arch. Hyg. Berl.,' vol. **56**, p. 341.
Strangeways and Oakley (1923–24). 'Proc. Roy. Soc.,' B, vol. **95**, p. 373.
Sulkowitch (1929). 'Johns Hopk. Hosp. Bull.,' vol. **44**, p. 439.
Trillat (1926). 'C.R. Acad. Sci., Paris,' vol. **183**, pp. 614, 1304.
—— (1927). *Ibid.*, vol. **184**, p. 170.
Wells (1931). 'Bull. Basic Sci. Res.,' vol. **3**, p. 5.
Wolfenden and Forbes-Ross (1900). 'Arch. Roentg. Ray,' vol. **5**, p. 3.
Wyckoff (1930). 'J. exp. Med.,' vol. **52**, pp. 435, 769.
—— (1932). 'J. Gen. Physiol.,' vol. **15**, p. 351.
Wyckoff and Rivers (1930). 'J. exp. Med.,' vol. **51**, p. 921.

The Ferrier Lecture

on

Problems Connected with the Principle of Humoral Transmission of Nervous Impulses

By O. LOEWI, Graz

(*Read June* 20, 1935)

Mr. President, Ladies and Gentlemen,—I am deeply appreciative of the honour, which has been done me by the President and Council of the Royal Society, in inviting me to deliver here, in this ancient and famous institution, a lecture which commemorates the work of Sir David Ferrier; and my sense of the honour is enhanced by the thought that my predecessors, as lecturers on this Foundation, have been Sir Charles Sherrington and Professor Ariëns Kappers.

Each of these lectures in memory of Sir David Ferrier is to deal with some subject related to the structure or function of the nervous system. Within recent years investigations in this field have dealt not only with the course of the specific nervous functions but, in an ever-increasing degree, with the analysis of nervous action into its fundamental factors, and especially those of a chemical nature. Having been mainly interested in these lines of development, and assuming that you have chosen the lecturer with a view to his own particular field of research, I have thought it proper that I should endeavour to deal with problems connected with the principle of the humoral transmission of nervous impulses.

I was the more ready to make this my choice of subject in that it may be taken to imply my appreciation of all the advances in this particular field of physiology, for which your country has been responsible. I need only remind you of such names as those of T. R. Elliott, W. E. Dixon, and H. H. Dale. Elliott suggested the conception; Dixon made the first experiment upon it; to Dale is due the greatest extension of its scope.

As you will know, in 1921 I succeeded in proving that, by stimulation of the nerves of the frog's heart, substances are released which, obtained in the fluid filling the heart and transferred to a test heart, produce exactly the same effects as the nerve stimulation. Thereby it was proved

that the nerves do not act directly on the heart, but that the direct effect of the stimulation of the nerves is to liberate chemical substances, and that the action of these substances is the direct cause of the characteristic modifications of the heart's action.

It seemed probable from the first that this mechanism of humoral transmission would not be found to be limited to this special case, but would be identified also in other organs. We shall soon see that this presumption was right. But first, in view of what is to follow, we must characterize the transmitters released by nerve stimulation. If I may start with the transmitter of vagus-stimulation, the "Vagusstoff," its effect, on account of its chemical instability, fades very quickly and can be annulled by atropine. Both properties are shared by the "Vagusstoff" with certain esters of choline, especially with acetylcholine. The quick destruction is caused by a specific esterase present in the heart. Furthermore, I was able to show that the action of this esterase is inhibited in a specific manner by minute amounts of eserine. This must be mentioned because it was this discovery which alone made possible the later extension of the proof of the occurrence of neuro-humoral transmission to a very wide range of effects. The "Vagusstoff" behaves exactly like acetylcholine, not only with regard to the properties already mentioned, but also with regard to each of its other peculiarities. Further, Dale and Dudley succeeded in isolating acetylcholine in substance from animal organs. To my mind these facts leave no doubt that the "Vagusstoff" is acetylcholine; I propose, therefore, to refer to it as such.

With regard to the characters of the substance released by stimulation of the cardio-accelerator nerves as well as by other sympathetic nerves, it could be shown that it shares many properties with adrenaline: both, for example, are destroyed by treatment with alkali and by ultra-violet and fluorescent radiation. The effect of both is inhibited by ergotoxine. Finally, as Cannon and Rosenblueth have pointed out, the action of the sympathetic substance is potentiated by small amounts of cocaine, in themselves ineffective, in exactly the same manner as Fröhlich and I demonstrated for adrenaline thirty years ago. In spite of all analogies, however, and although personally I am convinced of the identity, I do not feel justified as yet in assuming that the sympathetic transmitter is adrenaline, and I will therefore call it "the adrenaline-like substance."

And now we can proceed to discuss the extent to which the principle of the neuro-humoral mechanism is valid.

Firstly, it could be shown that the release of acetylcholine is to be observed, as the result of vagus stimulation, also in mammalian hearts.

Since the vagus belongs to the parasympathetic nervous system, many investigations have dealt with the question whether, and to what extent, the neuro-humoral mechanism can be detected in other parts of this system. Lack of time would prevent a discussion of the details of the results; it is, moreover, unnecessary, since Sir Henry Dale within the last year has given a full account of them in several lectures. So I need only summarize them by saying that there is no case known where the effect of parasympathetic stimulation is not due to the release of acetylcholine. Similarly with regard to the effects of sympathetic stimulation; just as in the heart, where, as we have seen, the adrenaline-like body is liberated, in nearly all other cases of sympathetic stimulation the release of an adrenaline-like body has been demonstrated. But, as Dale and his co-workers have shown, there are beyond doubt certain fibres belonging anatomically to the sympathetic system, the stimulation of which leads to the release not of an adrenaline-like substance but of acetylcholine. Dale therefore discriminates between cholinergic and adrenergic fibres, irrespective of their anatomical origin.

Hitherto a cholinergic function has been unquestionably demonstrated by Euler and Gaddum for the cervical sympathetic fibres, which cause flushing of lips and gums of the dog; by Bacq, for some of the sympathetic fibres responsible for contraction of the nictitating membrane; by Dale and Feldberg, for the sympathetic fibres causing sweat secretion in the cat ; and finally, by Buelbring and Burn for sympathetic vasodilators of the hind limbs of the dog. I have dealt with these results in a more detailed manner for the following reason. It may be known to you that W. A. Hess, and also Cannon, have looked at the sympathetic and the parasympathetic systems from a functional point of view. They regard the sympathetic as a system concerned with bodily activity, characterized by the fact that it innervates all actions, which augment the capacity of the organism for an immediate output of energy; while they regard the parasympathetic system as having the opposite type of effect, its function being to restore the lost energies. For these purposes the sympathetic usually acts by the release of adrenaline and of the adrenaline-like substance, while the parasympathetic causes release of acetylcholine. It seems to me that it might be a promising line of inquiry to attempt to discover whether, and how, the apparent exceptions to this rule, provided by the discovery of cholinergic sympathetic fibres, fit into the scheme of Hess and Cannon. Take, as an instance, the fact that stimulation of sympathetic ganglia related to the vessels of the hind limbs apparently causes the liberation of acetylcholine in the dog, but not in the cat; or that the secretion of sweat is evoked by the stimulation of cholinergic

sympathetic fibres in the cat, but of adrenergic sympathetic fibres in the horse.

However that may be, in this lecture the stress is to be laid upon the proof that the neuro-humoral mechanism is valid for the stimulation of both the parasympathetic and the sympathetic nerves; which means that it applies to the whole of the vegetative fibres, independently of whether they are adrenergic or cholinergic.

The question naturally now arises, what is the position with regard to the mechanism of stimulation of somatic nerves? There are some important investigations by Dale and Feldberg, which suggest the possibility that the stimulation also of spinal nerves is transmitted by humoral means, namely, by liberation of acetylcholine. Since these experiments are still in progress, I must content myself with a bare mention of this promising work, and will ask you now to consider what evidence there is for humoral transmission in the central nervous system.

When a substance is liberated by nerve stimulation, it is easily understood that the end result outlasts the stimulation itself for a variable time. The time is, within certain limits, dependent on the rapidity of liberation and on the amount of the substance as well as on the rapidity of its destruction or of its disappearance by other means—for instance, by diffusion. A prolongation of the effect beyond the period of stimulation is observed, not only in vegetative efferent nerve fibres but also in afferent fibres, the end organs of which are the nerve cells lying in the central nervous system. Further, as we know, an inhibitory stimulation previously applied may here have some influence on an excitation following relatively late, and although by itself ineffective, may shorten the period of a subsequent reflex tetanus. If we consider, in addition, that the central nervous system can effect a summation of applied stimuli—an effect made easily comprehensible by the assumption that each single stimulus produces a substance, and that repeated stimuli lead to an increase in its concentration up to the point of effectiveness—we can understand why, after the discovery of the "Vagusstoff," well-known nerve physiologists, among them Sherrington, Samojloff, Fulton, and Brücke, raised the question whether there might be a neuro-humoral mechanism existing also in the central nervous system.

Neuro-humoral transmission within the central nervous system is, of course, to be understood as the transmission of the excitation from one neurone to a second one, by means of a substance released at the synapse by excitation of the first neurone. There is no doubt that such an event within nervous structures is possible. Feldberg, Gaddum, and Vartiainen succeeded in demonstrating that by stimulation of preganglionic sympa-

thetic fibres acetylcholine is liberated at the synapse, and advanced the view that the ganglion cells, belonging to the second neurone, are excited to action by this acetylcholine, so that in this way preganglionic impulses are transmitted to postganglionic elements.

These facts are of fundamental importance, not only as indicating how, in a particular case, stimulation may be transmitted from a preganglionic to a postganglionic fibre, but, beyond this, by demonstrating for the first time that, in general, the transmission of excitation from one neurone to another may be of a humoral nature.

Though this instance gives nothing more than a demonstration of the transmission of impulses from one efferent neurone to another efferent neurone, the possibility is opened that, in an analogous manner, the transmission of excitation from afferent neurones to efferent ones, an essential feature of activity in the central nervous system, may be effected also by humoral means.

While there is not yet a distinct proof of substances being liberated by stimulating purely sensory fibres in the periphery, a beginning has been made of an experimental attack on the central nervous system in relation to our question. In A. J. Clark's laboratory, Dikshit ventured to compare the effect of central stimulation of the vagus with that of the injection of minute amounts of acetylcholine into the lateral ventricles. He found the effects of both nearly identical on the respiration and the heartbeat, the effect of vagus stimulation appearing after a certain latent period. Finally, it has been observed that during vagus stimulation in a few cases acetylcholine could be detected in the cerebrospinal fluid.

In this connection I should like to recall that, in accordance with the long-known fact that the mammalian heart-vagus ends before the ventricle, we found much more acetylcholine, both before and after vagus stimulation, in the auricle than in the ventricle, and that there was also more esterase in the auricle than in the ventricle. If it is permissible to draw from this the conclusion that the amount of esterase is higher where it is needed for the destruction of acetylcholine, then some importance may be attached to Plattner's observation that the brain is one of the organs richest in the esterase.

I should not regard all this as having an evidential value, but it may give at least a hint that humoral transmission may also occur in the central nervous system. Perhaps you will allow a pharmacological physiologist to dwell longer on mere possibilities in the central nervous system than on direct evidence concerning the periphery.

So much for the proofs, the likelihood, and the possibilities of the occurrence of the neuro-humoral mechanism. We must now discuss the

important problem, whether the nerve affects the function of its effective organ only by release of transmitters or whether it has, besides this, any additional function concerned in the effect of stimulation.

This matter can be settled by considering the mechanism of the action of atropine and ergotoxine respectively. We were able to prove, and it has been repeatedly confirmed by others, that these alkaloids do not, as was previously believed, paralyse the nerves themselves. Since the release of the transmitters still occurs in spite of the application of atropine or ergotoxine, these alkaloids only antagonize the effects of the transmitters. Since, after previous application of atropine or ergotoxine, as is well known, stimulation of the respective nerves is completely ineffective, it cannot be assumed that the nerve has any other effect on the function of the organ than to release the transmitters. What other kind of function, then, could remain for the nerve, since the effect of the released transmitters is identical with the entire effect of nerve stimulation, even to the smallest details? Though it is really obvious, I feel that it is necessary to express the conception in a formal proposition, as follows: in all cases where neuro-humoral transmission occurs, the dominion of the nerve extends only as far as the liberation of the transmitters: the spot where the transmitter is released is, in that sense, the effective organ of the nerve. From that point the dominion of the transmitter begins; the functional organ is therefore exclusively the effective organ of the transmitter.

Hitherto we have discussed only the question whether, in addition to the release of transmitters, the nerve has any other kind of influence on the specific function of its effective organ. We have just been able to answer that question in the negative; but there remains open the question whether the nerve has perhaps any other influence on the effective organ, apart from exciting its specific function. It is well known that denervated organs after a time become sensitized for certain directly applied stimuli. To this sensitization, according to general opinion, is due the frequent occurrence of at least a partial restoration of the function, which was earlier impaired. Take, as an example, the recovery of the blood pressure taking place some time after sympathectomy. One of the best studied cases is, perhaps, that of the dilator of the pupil, which becomes sensitized for adrenaline after extirpation of the superior cervical ganglion. What is known about the cause of this sensitization? If a substance necessary for the function of an organ has been absent for a long time, and later is again applied, the organ now reacts to it excessively. Since,

as we shall see, the transmitter disappears from decentralized or denervated organs, one might suppose that the sensitization of the denervated dilator for adrenaline, just mentioned, might be attributed to the previous disappearance of the adrenaline-like transmitter. But this explanation does not hold with the experience that, after sympathectomy, asphyxia by bleeding also causes a mydriasis of the denervated pupil, much more intense and of much longer duration than that which occurs in the innervated eye; and it holds still less for the following observation of Anderson. The sphincter pupillæ, after being decentralized by section of the oculomotor nerve, or denervated by extirpation of the ciliary ganglion, showed a marked sensitization for pilocarpine, this alkaloid being functionally to a certain extent similar to acetylcholine, but in other respects by no means equivalent to this transmitter; but, what is still more remarkable, there was also a striking sensitization of the sphincter of the pupil for asphyxia. The hypersensitivity for these stimuli can, of course, by no means be explained by the previous disappearance of the transmitters. I need hardly mention the fact, that the asphyxial miosis of the denervated pupil is not influenced by atropine. I should also like to mention another observation of Anderson; after previous decentralization or denervation of the sphincter, its tone returned after regeneration of the post or preganglionic tract, although no impulses passed by the regenerated nerves in response either to light or to direct stimulation of the fibres. Anderson concludes: "the regenerated nerves, therefore, exercised a trophic influence upon the sphincter before they allowed the passage of motor impulses under normal conditions." In a similar manner, I am inclined to consider the occurrence of hypersensitivity of the denervated organs as being due to a trophic disturbance caused by the denervation, although I am quite conscious that the term "trophic" is not in favour, because of its lack of precision. We have seen that the hypersensitivity always needs some time to develop; therefore it cannot be caused by disappearance of some kind of inhibitory central impulses, but it must be caused by the lack of a humoral effect, dependent on the presence of the intact nerve.

From these various considerations we can conclude that the nerve has two functions: the first is to initiate or to modify the specific functions of organs. This is performed only by the release of transmitters. The second, being continuously efficacious, is concerned not with the function but with the condition of the organs, and its effect is to lessen their sensitivity for directly attacking

stimuli. This latter function of the nerve, easily intelligible from the point of view of the consensus partium, is independent of the release of transmitters.

In what direction does the discovery of the neuro-humoral mechanism increase our knowledge beyond the experiences already discussed? To my mind the most important advance is that at last the old classical problem, concerning the nature of the transmission of the excitation from nerve to its reactive organ, has been conclusively answered. By the proof that this transmission is effected by substances liberated by the nervous system, evidence is given, for the first time, that the nervous system is not only the effective organ for chemical influences, and not only shares the general chemical metabolism of the body, but that, by itself, it exerts specific chemical actions in the organism. Is this a matter for surprise? Within the cell-complex of Metazoa which have no nervous elements, the relations between the cells obviously can be of no other than a chemical nature. Within the cell-complex of Metazoa having nervous elements, the nerve cell is not in principle different from other cells, but differs only, in accordance with its special purpose, in being provided with processes, the nerve fibres. From this point of view it was, perhaps, only to be expected that the relations between the nervous system and other organs would prove to be qualitatively of the same nature as the relations between the other organs, and would be found to have, like these, a chemical basis.

The next point of importance seems to me to be the disclosure of the nature of the peripheral inhibition. Hitherto it was not conceivable at all how stimulation of nerves can lead to an inhibition of the effective organs. Now that we know that it is brought about by release of chemical transmitters, the nature of inhibition is cleared up, and appears, indeed, to be self-evident. Since, at length, we know that the process of excitation in the nerve is, up to a certain point, quite unspecific; and since, in peripheral organs, an interference phenomenon concerned with the frequencies of stimulation cannot be considered as a possible cause of inhibition as it can, for instance, in the central nervous system, there remains, to my mind, no possibility whatever of imagining how nerve stimulation can inhibit an organ otherwise than by humoral means. In other words, the humoral mechanism presents the only conceivable mechanism of peripheral inhibition.

But further, this relevation of the nature of peripheral inhibition seems to be important also from the point of view of general physiology. The fact that, as in augmentory nerves, transmitters are liberated also by

stimulation of inhibitory nerves, means that, even with inhibitory nerves, the direct action of the nerve has the nature of a positive augmentation. Therefore, I do not hesitate to draw the important conclusion that in every case of nerve stimulation, whether augmentory or inhibitory in its ultimate effect, the direct effect is a functional increase, so far as the direct point of attack is concerned.

So much, or rather so little, about the fact, the extent, and the meaning of the neurohumoral mechanism. Having so far only touched on the general aspect, we must now proceed to questions of the more intimate mechanism.

What, for instance, is the localization of our transmitters, released by nerve stimulation? In other words, where is the point of the direct attack of the nerve stimulation? *A priori* there are two possibilities. The transmitters may be liberated either within the nerve-ending, or within the effective organ. The investigations hitherto concerned with this question have dealt only with acetylcholine.

We must first discuss, in this connection, certain observations on the acetylcholine content of denervated organs. Thirty years ago Anderson made the following statement: after degenerative removal of the ciliary ganglion light was for a long time ineffective, even on the eserinized eye. After a certain time, regeneration of the oculomotor nerve having evidently started, light again became active, but only after eserinization. While at that time a sufficient explanation of his observations was obviously impossible, the meaning of Anderson's results has now become quite clear. We know that stimulation of the oculomotor nerve releases acetylcholine. In the first stage after removal of the ciliary ganglion, acetylcholine having disappeared, eserine obviously ought to be ineffective. When the oculomotor nerve starts to regenerate, acetylcholine reappears, but in too small an amount to cause a miosis by itself, that is to say, without the protective and therefore potentiating action of eserine. Anderson's experiments, therefore, actually gave the first proof that the existence of acetylcholine in the eye is dependent upon the integrity of the ciliary nerves. Later Engelhart, in my own Institute, obtained direct evidence. He showed, by direct estimations made on the corpus ciliare and the iris, that the acetylcholine, to be found there in great amount when the nerve is intact, disappeared entirely after degeneration of the oculomotor nerve. Thereby it was demonstrated that, at least in some organs, the acetylcholine content and its maintenance are dependent on the presence of the nerves. What can be the nature of this dependence? I see only two possibilities. Acetylcholine either belongs to the nerve,

disappearing with its degeneration, or it belongs to the effective organ. If the latter be true we should be obliged to suppose that the formation and maintenance of the acetylcholine content in the effective organ are related to the nerve in some mysterious, trophic manner, so that it must disappear with the degeneration of the nerves, as Dale expresses the idea, "in some sort of atrophy of disuse." But an assumption that the transmitter belongs to the effective organ would need still further hypotheses. We should have to assume the existence of some mysterious particular transmitter system within the effective organ, distinct from the really effective organ, and, so far as I can see, without any analogy. For after degeneration of the oculomotor nerve the corpus ciliare and iris, which are its effective organs, do not degenerate at all, but nevertheless acetylcholine disappears. Therefore the influence of oculomotor degeneration would have to extend exclusively to this assumed transmitter system. A consideration of these difficulties alone establishes already a greater probability for the suggestion first mentioned, namely, that the transmitter belongs to the neurone, or more precisely to the nerve-ending. At least in the following case we have direct, and to my mind conclusive, evidence of the correctness of this assumption.

Mention has already been made of Feldberg and Gaddum's experiment, in which it was proved that stimulation of the preganglionic sympathetic fibres releases acetylcholine in the ganglion, which in its turn excites the gangion cells, so that these initiate the propagated impulses in the post-ganglionic fibres. These results have recently been confirmed by Gaddum for the inferior mesenteric ganglion. In very important experiments dealing with the question at which part of the ganglion the liberation of acetylcholine takes place, Feldberg and Vartiainen recently demonstrated that neither the preganglionic fibres in their continuity nor the ganglionic cells themselves, which are the only effective organ, release the acetylcholine. Therefore they conclude that the acetylcholine is released at the synapse. But a synapse is not an anatomical conception at all. It is a purely functional one, meaning the place where the nerve-ending makes contact with the cell. It is also used only in this sense by histologists, for instance by Greving, in the recently published volume of the "Handbuch für mikroskopische Anatomie." If it has been proved that acetylcholine is released at the so-called synapse, then, since the ganglion cell has been excluded, that can only mean, in my opinion, that the release occurs at the preganglionic nerve-ending. Though this has been proved, hitherto, only for the preganglionic endings of the sympathetic, several facts indicate that the transmitters are released from the nerve-endings in other cases also. We know that in some organs

not every functioning unit receives a nerve-fibre. According to Stöhr, for instance, there is at most only one nerve-fibre for every 100 capillaries; but when the nerve is stimulated all the capillaries react. From what point is the transmitter to diffuse, in such cases, to the non-innervated parts? In such a case there is, in my opinion, only the nerve-ending available as the place of release. Further, this assumption is supported by the consideration that in the most different kinds of organs, with their entirely different chemical structures and, therefore, very different types of metabolism, nerve stimulation leads always to the liberation of the same transmitters. Finally, it must be made clear that if the transmitters were not liberated at the nerve-endings, but somewhere in the effective organ, we should again have to postulate some mysterious, unknown mechanism transmitting the excitation from the nerve-ending to the effective organ; and then, to my mind, the discovery of the neuro-humoral mechanism would not amount to an advance in principle.

In this connection it is worth while mentioning that, in addition to the liberation of transmitters by nerve stimulation, histological observations make it probable that substances are also otherwise released by nerves. At the taste-buds the sensory nerve does not end endolemmally but epilemmally. In spite of this fact it has been known for a very long time that the taste-buds degenerate very soon after section of their sensory nerves. To my mind there can be no objection to the interpretation, which has been generally given to this effect, as due to a trophic influence, exercised by the nerve on the taste-bud by the mechanism of discharging a substance. In accordance with this view, the release of the transmitters may itself be just a special case of a more general faculty of nerves to release substances. This peculiarity of the nerves does not appear to be a matter for surprise; for the histological sketches of nerve-endings hitherto available show such a complicated structure, and are so different in different organs, that one can, without hesitation, attribute to them complicated functions.

Hitherto, we have been dealing only with the fact of the liberation of transmitters. And now, in dealing with the conception of the nature of nervous function, we reach the problem of the mode of this liberation. There are, so far as I can see, the two following possibilities to be considered, either the transmitters are not present within the nerve-ending unless the nerve is stimulated, being newly formed and released on stimulation, or they are already present within the unstimulated nerve-ending, and are simply rendered diffusible by the stimulation.

With regard to the ability of nerves, in any case, to produce transmitters, there can be no doubt at all that nerve is able to produce them. Years

ago, Witanowski demonstrated that acetylcholine can be found in the vagus, in the sympathetic chain, and in sympathetic ganglia. It cannot have diffused there either from the blood, where it is not present, or from other tissues on account of its rapid destruction. From this we may conclude that nervous structures, as such, are in principle able to form acetylcholine. But it is quite another question whether the nerve builds up the transmitters in immediate response to stimulation. Obviously it is impossible to demonstrate such a production of substances in the nerve-endings themselves; one can for this purpose investigate only the whole organ. This is a drawback, since the transmitter, at least in some organs, is certainly not confined to the nerve-endings. For instance, as has been shown by Dale and Dudley, and by Chang and Gaddum, acetylcholine, and indeed in very great amount, has been found not only in organs where, as in the spleens of cattle and horses, we have no reason to assume a particularly rich nervous supply but also in an organ entirely lacking a nervous supply, such as the human placenta. The amount of acetylcholine not connected with the nerve-endings may sometimes be so great that an increase on nerve-stimulation, if it occurred, could not be detected analytically. That may be one reason why hitherto an increase with nerve stimulation has been demonstrated only in certain organs, and why, even in the same organ, the results of different investigators differ among themselves. Altogether, the number of researches on this point is as yet too small to justify a generalization regarding the formation or the non-formation of transmitters on nerve-stimulation in the different organs; moreover, experiments of this kind concerning the adrenaline-like transmitter have not yet been made at all.

But while it would be of interest to know whether nerve-stimulation does lead to a new formation of transmitters, this question does not seem to be of such a fundamental importance. The essential and established effect of nervous stimulation is to liberate the substances and to make them diffusible, whether they were already present or whether they are newly formed.

The alternative possibility mentioned above, that nerve-stimulation may act by liberation and mobilization of the substance already present in the unstimulated state, necessitates the assumption of the presence of the transmitter in a non-diffusible form. What we know as yet as to this possibility is concerned only with acetylcholine. In the absence of vagus stimulation the heart contains acetylcholine in an amount sufficient to stop the heart, if it were freely diffusible. In fact, it is without action and, in contrast to acetylcholine in diffusible form, it is protected against destruction by the esterase. In experiments which I did with Engelhart

we found that the acetylcholine content of a heart does not decrease during many hours, provided the heart is kept intact. Dale and Dudley made the same observation with the spleen. This means that in the intact organ the esterase has no influence on the acetylcholine; but free acetylcholine, added to the intact heart, will be destroyed as being diffusible. Therefore, in agreement with Dale, I take it to be obvious that acetylcholine in the intact organ is present in some labile, non-diffusible, perhaps adsorptive combination, and that in this combination it is neither active nor liable to attack by esterase. There is evidence that this combination must be extremely labile, since the mere destruction of the physical integrity of the cells, for instance by grinding the organ, is sufficient to cause the acetylcholine to be split by the esterase within a very short time. Similarly, nerve stimulation might so act as to liberate the acetylcholine or the adrenaline-like body, present in the nerve, from its combination. That might be brought about, for instance, by the nervous stimulation making surface-active substances effective. Experiments which I have already started seem to suggest that there are surface-active substances which act in the heart in a manner resembling the known action of surface-active substances in serum. Serum holds free alkaloids, if these are added to it, in a non-diffusible combination; a surface-active substance dislodges them and makes them again diffusible.

You will have seen that the question, whether the transmitters liberated by nerve-stimulation are newly formed or merely split off from a combination, is still an open one. I think, however, that the function of a lecture is not only to reproduce accepted facts but also to direct attention to open problems.

The lapse of time between stimulation of the nerve and the response of the reacting organ is an extremely small one, considering that there must be at least three steps involved in it: liberation, diffusion, and action. The time of delay varies in different cases, being partly, no doubt, dependent on the distance of the liberating nerve-ending from the effector cell. At the ganglionic synapse, according to Brown and also to Eccles, it amounts only to 2 σ. That shows that the release is performed at the very moment of the stimulation. The further fact that the effect on the ganglionic cell is also brought about practically without delay is convincingly explained by Dale, by the liberation at the nerve-endings taking place in direct contact with the ganglionic cell. In the heart the delay is also absolutely small, since already the first contraction following the onset of vagus-stimulation is diminished; but since it amounts to 80 to 100 σ, according to Eccles, it is long in comparison with the delay at the

ganglionic synapse. In the heart a significant time for diffusion to the effector cells is required. The rapidity of disappearance by destruction or diffusion, and therefore the persistence of the effect after the stimulus, also differs in different organs. The discovery of the humoral mechanism of vagus-stimulation on the heart would not have been possible if the destruction of acetylcholine had not, in this case, occurred so slowly that there was time enough for it to diffuse in effective amounts into the liquid filling the heart. In the ganglion, on the contrary, the destruction is performed so rapidly, that acetylcholine can only be found if the perfusion fluid contains eserine. The differences regarding the time-relations in these typical instances are easily to be understood by considering the different functions to be served and the different types of transmission required in the ganglion cell and in the heart respectively.

Following the natural sequence of events, we now proceed to discuss the exact point of action of the transmitters. Before it was known that the vegetative nerves, on stimulation, release substances responsible for the effect of nervous stimulation, it was quite generally assumed that the so-called vago-mimetic and sympathomimetic agents stimulate the nerves somewhere peripherally. There were two reasons for this assumption. Firstly, their effect is identical with that obtained by stimulating the respective nerves. Secondly, it was believed that the action of the alkaloids which paralyse the effects of the above agents, namely, atropine and ergotoxine, was to paralyse the respective nerves. There was, on the other hand, a difficulty in assuming that these agents stimulated nerves since they were effective even after the nerves had degenerated. In order to overcome this difficulty the assumption was made, and was doubtless justified at the time, that the point of action of these neuromimetic substances was a myoneural junction not degenerating on nerve-section. This assumption can now no longer be maintained. The transmitters, being vagomimetic and sympathomimetic agents, would have to act in the same manner, namely, by stimulating the myoneural junction, and there causing liberation of transmitters, that is of themselves, and so on. But how then could the effect on the effector organ be produced ? Besides this logical impossibility, the assumption of the transmitters stimulating the nerve at any point was made entirely superfluous by the evidence quoted above, showing that the alkaloids atropine and ergotoxine, in inhibiting the effect of the vagomimetic and sympathomimetic substances, do not, as was previously believed, paralyse the nerves, but are mere antagonists of the transmitters. Moreover, acetylcholine and adrenaline, and therefore, of course, also the adrenaline-like transmitter, are also effective on organs which are entirely without nervous supply. Acetyl-

choline, for instance, dilates vessels which are not innervated from the parasympathetic. Adrenaline and, therefore, doubtless, also the adrenaline-like transmitter, on the other hand, increases, for instance, the activity of the embryonic heart at a stage at which it lacks nerves; and so on. It is therefore not the nerve which is the point of attack of the transmitters but some part of the reacting organ, which is probably not differentiated morphologically but only chemically or physico-chemically.

To adopt an expression which Dale has used, we can no longer say that the transmitters reproduce the effects of the nerves; we must state rather that the nerves reproduce the effects of the transmitters, because the nerve impulses liberate the transmitters which, in their turn, directly produce the effects.

It is remarkable that a particular transmitter, when artificially applied, should act just at the points where it is normally liberated by nervous impulses; that is to say, it is exactly there that the effector cells are specifically prepared for its action. To my mind this phenomenon cannot yet be explained causally. We must rather look upon it as having an analogy with that specific sensitivity of certain organs for special chemical agents which, at present, we can only understand teleologically; take as one instance the sensitivity of the respiratory centre for CO_2. This well-known sensitivity we encounter everywhere within the living organism, as one of the fundamental factors of its ability to function, and therefore to exist.

Hitherto, we have been dealing with the effects of the transmitters on the particular organs in which they are liberated. Do they act only there, or also in other remote organs? Since the transmitters to a certain degree pass into the blood, one must admit in principle the possibility that their effect is not restricted to the stimulated organ, but may also spread to remote organs. It seems to me that such a distant action is improbable under normal conditions. Why, in a given case, where only a retardation of heart action is needed, should a concomitant intestinal colic take place? At the same time, it cannot be excluded that it may sometimes happen—under pathological conditions, I should say; especially as we have evidence that it does occur under special experimental conditions. If, for instance, the esterase action is inhibited by previous administration of eserine, acetylcholine passes in sufficient amount into the blood to reach other organs and act upon them. Furthermore, Cannon, by previous sensitization of organs, rendered them hypersensitive for the adrenaline-like body to such an extent that it, though liberated in quite another organ, exerted its influence upon them. Similarly, it may happen in pathological conditions, either through over-production,

or through insufficient destruction, or through hypersensitivity of organs, that a remote effect may be caused. It would be desirable that, in future, clinicians should keep an eye upon this possibility, in order perhaps to find better explanations for certain symptoms hitherto considered to have a purely reflex origin.

Under physiological conditions, however, the body has at its disposal the hormones to influence the organs generally.

Finally, although it carries us a little beyond the field of humoral transmission as such, I may be allowed to raise the question whether in addition to the difference in the localization of the action of transmitters, often called local hormones, on the one hand, and of true hormones on the other, there is also a difference in character between their respective actions. Such an inquiry would obviously be of value for an analysis of the interference of nervous and humoral factors in the organism, disclosing their respective roles.

Generally speaking, transmitters are released whenever the specific, acute action of an organ is to be initiated or modified. In quite the same manner act the hormones of two of the endocrine organs - adrenaline produced in the suprarenal medulla, and vasopressin and oxytocin in the posterior lobe of the hypophysis. Thus, only these out of all the hormones can be tested in an acute experiment, like the effects of nerve stimulation. It is obvious that adrenaline is very similar to, or even identical with, the sympathetic transmitter. In agreement with this similarity in the mode of action of their products, the suprarenal medulla and the posterior pituitary lobe differ from the rest of the ductless glands by peculiarities common only to them; the development and the condition of both of them seems to be independent of the anterior pituitary lobe. Further, the secretion of both of them seems to be initiated by purely nervous means. For adrenaline we know that its physiological secretion is not initiated by any hormonal stimulus. In the posterior pituitary lobe, although our knowledge regarding the events occurring there is much less complete hitherto, at least we also know only of a nervous initiation of the secretion of vasopressin and oxytocin, namely, by electrical stimulation of the hypothalmic region.

Finally, the suprarenal medulla, as well as the posterior pituitary lobe, are organs of a neurogenic character; the medullary cells of the suprarenals are modified ganglionic cells, the posterior lobe cells are related to glia cells. According to Alfred Kohn, the posterior lobe is just a part of the brain, functionally united with the hypothalamus. Both glands accordingly represent modified parts of the nervous system.

All the other endocrine organs and their hormones behave in quite a

different way. As to their hormones, they generally do not influence the specific acute action itself but merely the conditions of the specific functions of the organs—their development, state, and metabolism. Further, their secretion is not initiated only in accordance with requirement by nervous means, but goes on continuously and automatically, being only under hormonal or some other kind of chemical influence. Only in some cases, as in the pancreas and thyroid glands, is a nervous control also to be found. As regards their development, their state, and, partly, their function, they are not governed by the central nervous system but by the anterior pituitary lobe. This regulating gland, in contrast to the central nervous system, receives information about the needs of the organism not, so far as we know, by nerves but almost exclusively by humoral means, and responds to these needs in the same way.

Therefore in general we can see a division of labour between nervous and hormonal events, and accordingly between the respective regulators, the central nervous system, and the anterior pituitary lobe. The central nervous system regulates principally the specific, acute functions; therefore it also influences those neurogenic endocrine organs, the adrenal medulla and the posterior pituitary lobe, the hormones of which cause acute changes. The regulator of the non-neurogenic hormonal system, the anterior pituitary lobe, regulates mainly the development and state, and partly also the secretion, of the remaining endocrine organs, the hormones of which bring about longer lasting changes of the conditions of many other organs; take, as an example, the action of the growth hormone of the anterior lobe. On the whole, we may be allowed to summarize, with a little dogmatic simplification, by stating that the central nervous system, being the representative of the nervous functions, is the regulating centre of a c t i o n s, the anterior pituitary lobe being the representative of the hormonal functions, is the regulatory centre of s t a t e s.

With this summary we can also answer the above question, as to the difference between the roles of nerves and their transmitters on the one hand, and of the hormones on the other, by stating that there exists not only a difference of localization but also a difference of character between these two groups of actions.

A characteristic of all living substance is its ability to react to stimuli. The more we progress in our knowledge of the nature of stimuli, the more the evidence increases that stimuli of a chemical nature are predominant. Who would have thought, years ago, that mechanical stimuli may act indirectly by producing effective chemical agents, as for instance Sir Thomas Lewis has pointed out so impressively? And who

would have thought, years ago, that nervous stimulation influences the organs by releasing chemical substances, and that by such means the propagation of impulses from one neurone to another is effected?

The study of the effect of chemical agents on the organism is the main problem of pharmacology. Therefore it is not astonishing that that line of research, in particular, which deals with the disclosure of the chemical mechanism of nerve stimulation has been inaugurated by pharmacologists, and that pharmacologists in the main have worked it out. Being myself a member of this guild, I may, in the interests of physiology as well as of my own field of activity, close with an expression of the hope that the pharmacological department of neurophysiology may widen yet further its scope, especially in the direction of the central nervous system.

616—016.42 : 612.014.481

On a Method of Increasing the Sensitiveness of Animal Tumours to X-Rays

By S. Russ and G. M. Scott

(Barnato Joel Laboratories, The Middlesex Hospital)

(*Communicated by C. H. Browning, F.R.S.—Received May* 16, 1935)

Any means of increasing the sensitiveness of human tumours to X-rays or gamma rays would certainly change the outlook on the treatment of cancer in many sites, more especially for the types of tumours which are sometimes termed radio-resistant. The experiments recorded here were mainly carried out with a fairly rapidly growing sarcoma of the rat (F.16), originally given us by the Imperial Cancer Research Fund in 1921. We (1933) have already referred to the modification which it has since undergone, particularly to the extraordinary increase in its rate of growth and cellularity of structure. These changes are probably due to the technique used in its transplantation, and to the use of a strain of rats* particularly sensitive to tumour implantation. With Jensen's rat sarcoma, 100% of progressive tumours is generally obtained from grafts and 90% is looked upon as a rather poor result. Another obvious advantage for experi-

* These rats, which are of black and white breed, originally came from Dr. Hartwell, King's College, Campden Hill, and they have now been bred in our laboratories for several years.

ments of the type described here is that spontaneous absorption of an established tumour of either F.16 or J.R.S. is extremely rare.

In the first experiments established tumours were exposed for varying lengths of time to carefully measured doses of X-rays generated at about 170 kV and filtered by 10 mm of aluminium. The minimum amount of radiation necessary to cause the disappearance of *all* tumours of each type is referred to here as the "lethal" dose. It is expressed by time—for sarcoma F.16 it is 200 minutes, approximately equivalent to 1000 r. A dose of X-rays amounting to 30–40% of the lethal dose generally has some deterrent effect on the growth of the tumour; 40% of the lethal dose very rarely causes the disappearance of a well-established tumour. The effect becomes more definite and lasts longer as the dose is increased and the majority of tumours disappear after a dose which is 75% of the lethal dose. Great care has always been taken to expose only the tumour, as it has been observed that the dose of X-rays required to cause complete disappearance is considerably less when even a small area of surrounding tissue is irradiated. The actual size of the tumour at the time of irradiation also influences the final result; quite small, young tumours may disappear with a comparatively small dose of X-rays, while very large ones are sometimes little affected by a much larger dose, in spite of strict attention to the principle of dosage which is measured by the ionization at the lowest level of the tumour. Most of the tumours used for these experiments were between 16 mm and 22 mm in diameter at the time of exposure to X-rays.

With the above data as a basis for experiment, another group of rats bearing established tumours was injected with chemical substances before exposing the tumours to sub-lethal doses of X-rays, usually 80 minutes, *i.e.*, 40% of the lethal dose (*see* above). The interval between this injection and the X-ray exposure was generally 24 hours, but in a few cases it was as much as 5 days. Three of the substances used belong to the group which produces vital staining, viz., vital new red (Cappell, 1929), trypan blue (C.I. No. 477), isamine blue (C.I. No. 710), the fourth was thorotrast.* Control experiments showed that these substances given under the same conditions had little effect, if any, on the subsequent growth of the tumour. The experiments fell into two groups: (*a*) those in which the substance was injected subcutaneously in the flank opposite that in which the tumour was growing; and (*b*) those in which the injection was made directly into the tumour itself.

* A preparation of thorium dioxide in sterile ampoules supplied by Chemische Fabrik von Heyden Aktiengesellschaft, Dresden.

Experimental Details

Vital New Red—Nineteen rats with growing grafts of sarcoma F.16 were given an injection of 5% solution of the dye in physiological saline on the opposite flank; the dose was at the rate of 1 cc per 100 grams body weight. The results, Table I, showed that the dye did not increase the susceptibility of the tumour to X-rays.

Table I

X-ray dose in minutes of exposure	Other treatment	Number of tumours treated	Number of tumours which disappeared
80	Nil	36	3
80	Rats inoculated in the flank with 5% vital new red	19	2
50	Rats inoculated in the flank with 1% trypan blue	6	3
80	,, ,,	17	11
50	Tumours inoculated locally with 0·2% trypan blue	2	0
80	,, ,,	28	10
80	Tumours inoculated locally with 1% trypan blue	23	12
50	Tumours inoculated with 0·1–0·3 cc thoro-trast	7	2
60	,, ,,	20	8
80	,, ,,	15	8

Trypan Blue—Twenty-three rats with growing tumours of sarcoma F.16 on the opposite flank were given a 1% solution in saline at the rate of 1 cc per 100 grams body weight. Eighteen of these tumours were irradiated on the day after the injection of the dye; for the remaining 5 the interval was 2 or 3 days. The results showed that tumours in the vitally stained rats were considerably more sensitive to X-rays than those in the unstained rats after the same dose of X-rays, as 65% of the blue tumours disappeared, compared with 8% of the unstained.

In another series the dye was injected directly into the tumour, the dose being at the rate of 0·5 cc of a 0·2% solution for a tumour measuring 20 mm in diameter; the total dose of dye in these cases was rather less than one-tenth of that in the previous group. The susceptibility of the tumours to irradiation appeared to be somewhat increased (36% dis-

appeared). As many of the tumours so treated, after nearly disappearing, began to grow again, a further series was similarly treated, except that the strength of the dye was increased to 1%; this led to the disappearance of 12 out of 23 tumours after irradiation.

Thorotrast—The tumours of 42 rats received a local injection of this substance at the rate of 0·1–0·3 cc for a tumour measuring 20 mm. These tumours were afterwards exposed to varying doses of X-rays, Table I. The results show that their susceptibility had been increased. As thorotrast is relatively opaque to X-rays, radiographs of the tumours which had been injected were made before treatment. These showed great variation in the distribution of the substance, which in some tumours was very uneven. In these cases the tumours eventually grew in spite of irradiation, while most of those which showed more even distribution of the thorotrast disappeared after similar X-ray treatment. Radiographs of rats taken as long as 16 months after the injection of thorotrast still showed it at the original sites.

Isamine Blue—Rats bearing tumours of Jensen's rat sarcoma were injected locally with a 1% solution of the dye in distilled water at the rate of 1 cc for a tumour 20 mm in diameter. As this tumour is more resistant to X-rays than F.16, exposures of 100 and 120 minutes were given. The results showed no obvious alteration in the sensitiveness of the tumour as 2 out of 7 disappeared after irradiation for 100 minutes, both in the series which had received dye and in the unstained controls. Similarly, of 11 stained tumours exposed for 120 minutes to X-rays 4 disappeared, and of the unstained controls irradiated simultaneously 4 out of 11 also disappeared.

In this connection we may refer to the work of Roosen (1930), who claims that the injection of isamine blue has a definite inhibiting action on the growth of human tumours. Bernhardt, in the same year, claims that by means of combining X-ray doses with intravenous injections of "Isaminblau 6B" he was able to observe considerable improvements in the results of treatment of a large number of patients suffering from various forms of cancer. The doses of isamine blue which we used subcutaneously do not appear to have had any inhibitory action on the growth of the tumour.

Conclusions

From experiments on over 200 tumours it appears that well-established, growing tumours of rat sarcoma (F.16), vitally stained with trypan blue, injected either into another part of the body or locally into the tumour,

show marked increase in sensitiveness to the effect of X-rays. After irradiation for 80 minutes the tumours of only 8% of the control unstained animals disappeared, while of those treated with dye 36 to 65% disappeared. Thorotrast injected into the tumour has a similar action. Under similar conditions staining with vital new red or isamine blue did not affect the susceptibility of the tumours to irradiation.

There has been no apparent toxic effect, at the time of injection or later, from either thorotrast or trypan blue used in these experiments. The rats which were given the small general dose of trypan blue remained blue for several months, and those injected with thorostrast still showed it at the original site a year later, but otherwise appeared to be in good condition. The dose of trypan blue per body weight was much smaller than that used by Ludford (1932), which produced toxic effects on mice.

We are indebted to Professor C. H. Browning, F.R.S., for suggesting the actual substances under test in this work.

References

Bernhardt (1930). 'Med. Klinik,' vol. **26**, p. 83.
Cappell, D. F. (1929). 'J. Path. Bact.,' vol. **32**, p. 595.
Ludford, R. J. (1932). '10th Sci. Rep. Imperial Cancer Res. Fund.'
Roosen, R. (1930). 'Krebsforsch.,' vol. **31**, p. 506.
Russ, S., and Scott, G. M. (1933). 'Brit. J. Radiol.,' vol. **6**, p. 451.

575 · 242

A New Sub-Lethal Colour Mutation in the House Mouse

By Hans Gruneberg

(Department of Zoology, University College, London)

(*Communicated by J. B. S. Haldane, F.R.S.—Received May* 24, 1935)

[Plates 19–21]

1—Introduction

In this paper, a new gene is described in the house mouse which arose, presumably by a spontaneous mutation, in one of our stocks. The genetics of the case are rather simple and can be dealt with shortly. The main attempt is to show how manifold may be the effect of one single Mendelian factor, and to demonstrate that the primary effects caused by a factor lead to a chain of secondary and tertiary alterations which, although ultimately caused by that hereditary unit, are only associated with it more or less mechanically. Since we usually see only the more remote consequences of the action of a gene, the goal of such an investigation should be to trace all secondary and tertiary actions back to that stage in development to which all the manifold effects converge, the original starting-point of an anomaly. In other words, we have an experiment in embryology in a reversed direction; while in experimental embryology the experiment is known, but the result is doubtful, here we have to study the results first and by going backwards to find out what sort of an experiment nature has performed.

Since this process is long and laborious, only the very first steps can be demonstrated here. This paper is confined to the investigation of bones and teeth. In a later communication the author hopes to be able to deal with the inner organs, embryology, and some physiological problems which arise from this work.

2—Origin and Genetics of the Mutation

The new gene to be described below was found in an extreme-dilute stock derived from Dr. L. C. Dunn's laboratory. Extreme dilution is an allelomorph of albinism which, in a mouse carrying the wild-type allelomorphs for all other factors, dilutes the coat to a dirty white or very

faint grey and the eyes to a very dark ruby, almost black colour. Since females of this type are slow breeders, the stock was not kept homozygous for this gene, but homozygous males were crossed to heterozygous females, or two heterozygous animals were crossed together. The presence of the wild-type allelomorph of extreme dilution (c^D) in some of the animals proved fortunate, since otherwise the new type would probably have escaped our attention.

The first mutant animal was observed in a litter derived from a pair heterozygous for c^D. The animal was remarkable from the fact that its coat contained not a trace of yellow pigment but was pure grey. In this respect it resembled chinchilla (c^{ch}), another allelomorph of albinism, the main difference being that chinchilla not only suppresses the yellow pigment, but also dilutes the black pigment to some extent; $c^{ch}c^{ch}$ has thus a similarly pure but lighter grey coat. Since the animal was derived from parents which were known to be heterozygous for the albino allelomorph c^D, it was clear from the very beginning that the new factor could not belong to that series.

The animal in question was smaller than its sibs and was growing at a much slower rate. Eventually growth stopped altogether while the normal litter mates showed no retardation. The eyes of the little creature were opened about a day later than those of the normals. After a few days the eyelids had a tendency to stick together, which was caused by some discharge from the eyes. This condition changed from day to day, affecting the eyes sometimes unilaterally, sometimes bilaterally. The general physiognomy was fundamentally changed by the fact that the whole snout was considerably shortened. So the combined differences of coat colour, size, half-closed eyes, and dumpy snout made the distinction from the normal sibs quite easy. In its fourth week the animal died after having obviously lost weight during the last days.

From the same parents, another young one of the same kind was obtained, which showed exactly the same characteristics and also died at about the same period. Larger stocks have since been established by testing sibs of the new mutant for heterozygosity for that factor. Altogether 150 animals of this type have been found: all showed the same complex of symptoms and they all, with some exceptions to be mentioned below, died between the 22nd and the 30th day of their lives. Thus there can be no doubt that the new factor is associated with a lethal effect. From the pure grey colour and this effect I have called the new form "grey-lethal" (symbol *gl*).

Grey-lethal behaves as a recessive factor. Animals heterozygous for it are indistinguishable phenotypically from the normal. Tables I–IV

show the results of some crosses; in Table I grey-lethal and extreme dilution are both segregating as an F_2; Table II shows an F_2 for *gl*, but a back-cross for c^D, while in Table III only *gl* is segregating, either on intense or on extreme dilute background. In the first period of this work, grey-lethal could be detected in intensely coloured animals but not in the presence of extreme dilution; since c^D suppresses all the yellow and most of the black pigment, no alteration by the action of *gl* was to be expected, and in fact an animal which is an extreme dilute + grey-lethal is not different in coat colour from an ordinary c^D. Thus the first c^D mice of these experiments were not recorded for *gl*; later, as will be shown below, other characters allowed for the detection of grey-lethal on any coat colour.

In another experiment, an intense agouti female heterozygous for grey-lethal and extreme dilution was outcrossed to a black non-agouti male (hairs lack the yellow tip). By inbreeding their offspring so far as it proved to be heterozygous for grey-lethal, the figures of Table IV were obtained. It was found that a black non-agouti grey-lethal is just as black as an ordinary non-agouti mouse, so that it cannot be distinguished from it by its coat.

TABLE I

$$\frac{gl}{+}\frac{c^D}{+} \times \frac{gl}{+}\frac{c^D}{+}$$

Intense		Extreme dilute		
Normal	Grey-lethal	Normal	Grey-lethal	Unclassified
89	28	26	6	20

TABLE II

$$\frac{gl}{+}\frac{c^D}{c^D} \times \frac{gl}{+}\frac{c^D}{+}$$

Intense		Extreme dilute		
Normal	Grey-lethal	Normal	Grey-lethal	Unclassified
72	31	71	18	7

TABLE III

$\frac{gl}{+} \times \frac{gl}{+}$ (families not segregating for c^D)

	Normal	Grey-lethal
On intense background	71	19
On extreme dilute background........	55	20
Total..................	126	39

TABLE IV

$\frac{gl}{+}\frac{a}{+} \times \frac{gl}{+}\frac{a}{+}$ (in a few litters $\frac{gl}{+}\frac{a}{+}\frac{c^{D}}{+} \times \frac{gl}{+}\frac{a}{+}\frac{c^{D}}{+}$)

Intense				Extreme dilute	
Agouti		Non-agouti			
Normal	Grey-lethal	Normal	Grey-lethal	Normal	Grey-lethal
63	18	19	7	6	3

The figures show that grey-lethal behaves as a simple recessive factor segregating independently of the albino and of the agouti series. Taking all the data together, there are 472 gl: 150 gl, which is a good approximation to a 3 : 1 ratio. There is no indication in the figures of an increased pre-natal mortality of the grey-lethals.

3—General Development of the Grey-Lethal

In order to get more information about the nature and cause of the lethal effect, the growth-rate of the grey-lethals was followed up more thoroughly.

Animals were marked as quickly after birth as possible and weighed at 24-hour intervals up to the death of the grey-lethals. The mother was left with the litter all that time. The food of the mother was that generally used in our laboratory and consisted of dog biscuits, wheat, oats, carrots, and water from a drinking-bottle.

TABLE V—EARLY WEIGHTS OF FOUR LITTERS

First weights taken	Normal young	Grey-lethals	Died unclassified
Day of birth	0·95 1·11 1·13 1·17 1·27	0·91 0·97	1·08 1·11
Day of birth	1·60 1·78 1·83	1·63	—
About 24 hours after birth	1·38 1·39 1·43 1·46 1·54 1·66	1·23	—
About 36 hours after birth	1·91 1·98	1·21 1·75 1·87	1·02

It was found that on an average the new-born grey-lethal is already somewhat lighter than the normal litter mates; some overlapping between the birth weights occurs, as shown in Table V.

Fig. 1 shows the individual growth-curves of two normals and two grey-lethals out of a litter of seven young; the curves of three other normals which showed no essential difference are omitted for sake of clarity. The curves of the normals show a steady gain of weight with some slight

irregularities at weaning age (about 20th–21st day). The two grey-lethals gain weight fairly normally during the first week; they continue to gain in the second week, but somewhat more slowly than the normals. Beginning with the 15th day, the weight remains almost constant for some time, then the curves decline until eventually the animals die. Essentially the same type of curve has been obtained several times. The best conditions seem to be when a litter contains several grey-lethals and only a few normals. In this case there is obviously less competition for

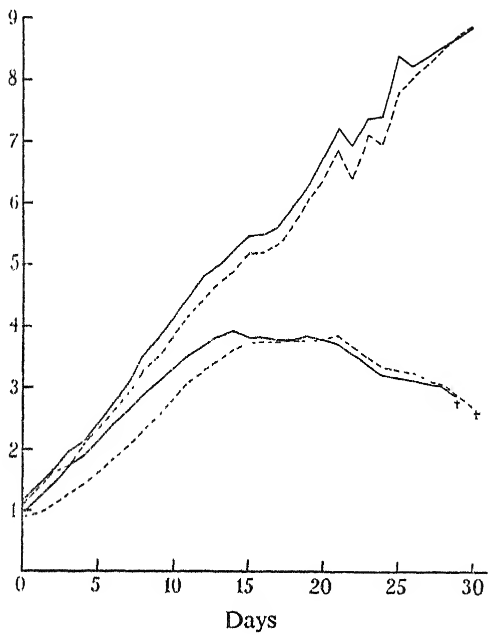


FIG. 1—Growth-curves of two normals (above) and two grey-lethals (below) out of a litter of seven. Weight in grams

the mother's milk, and the grey-lethals may gain weight over a longer period; this, however, does not increase the length of their life, but only shortens the period of weight constancy.

The eventual loss of weight in the grey-lethals coincides with the age at which the young (or rather, the normal young) are weaned. Actually, I observed that the grey-lethals continued suckling even during the fourth week. This led to the assumption that the shape of the weight-curves of the grey-lethals during the last period of their life is the expression of a decline in the mother's lactation after she has weaned the normal young. To test this, the mother was taken from the litter at the 20th day after birth, viz., the weaning age of normal young. The result is given in fig. 2.

The result is quite clear; immediately the mother is taken from the litter the grey-lethal declines rapidly and dies in a few days. The normal litter mates, although obviously somewhat disturbed at that time, are still alive now and are already grandparents. The experiment shows that the presence of the mother is essential for the continuation of the life of a grey-lethal.

At this stage of the experiment, a negative observation gave the clue. While normal young mice are often seen nibbling solid food even before the actual weaning, I could not remember ever having seen any grey-lethals nibbling. This made me inspect their mouths. Not a single tooth was visible. The anatomical and histological details will be dealt with in the next sections. In this connection, this fact gave the explanation for the result shown in fig. 2. The sudden decline and quick death means complete starvation. The slower decline, as shown in fig. 1, seems to be partial starvation; the mother weans the normal young, presumably since their growing teeth are painful to her, and the few remaining grey-lethals which are still suckling cannot keep lactation going; so it gradually declines, sealing the fate of the grey-lethals.

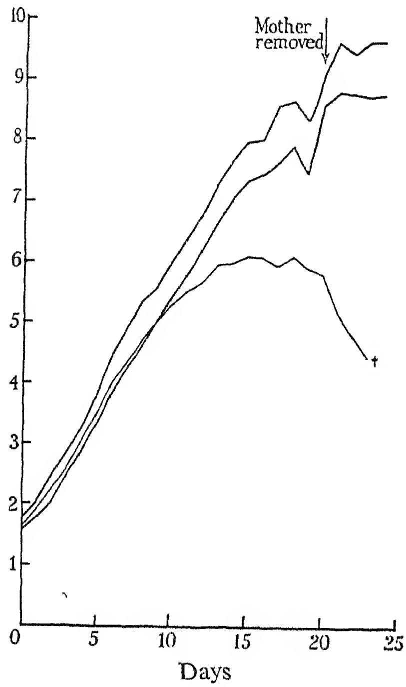


FIG. 2—Growth-curves of two normals (above) and one grey-lethal out of a litter of four. Effect of weaning

Now the question arose whether the difference in the growth-curves of normals and grey-lethals is only caused by the fact that the normals can make use of their teeth by taking solid food in addition to the mother's milk which the grey-lethals cannot do. In other words, is the death of the grey-lethals caused purely mechanically by the absence of teeth, or are endogenous causes responsible? The latter seemed more likely, since there are already some differences in the growth-curves at an age when the young are certainly only dependent on the mother's milk. At any rate, the relative importance of endogenous and mechanical causes had to be determined.

To test this, normal litter mates had to be kept under such food conditions that their teeth gave them no advantage over the grey-lethals.

Accordingly, the diet was changed completely. The mother was given a mixture of cow's milk, eggs, glucose, and a few drops of cod liver oil from a dish, and in addition received ground oats (Robinson's Patent Groats). When the young grew older all the normals and some of the grey-lethals learnt to feed from these dishes quite readily, and the normals had now no advantage whatever over these grey-lethals. The diet was

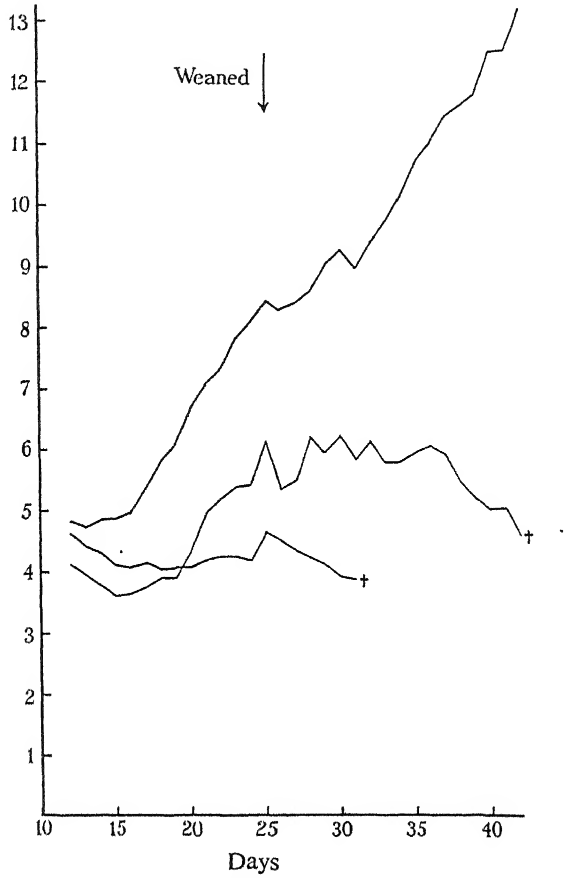


FIG. 3—Compound growth-curve of three normals (above) and individual growth-curves of two grey-lethals. Finely divided food. The longer-lived grey-lethal fed very well all the time, while the other individual was a bad feeder

changed on the 13th day, which was early enough, since the young do not start taking solid food before that day. The result of such an experiment is given in fig. 3.

Immediately after the change of diet there is some slight decline in all the animals; obviously it takes a day or two before the mother gets used to the new kind of food. After this short decline, however, the normals

gain weight steadily, thus demonstrating that the food as such is suitable. On the other hand, the grey-lethals also gain, but much more slowly; eventually the weight becomes constant, then declines, and finally the animals die. The maximum age reached by a grey-lethal under these food conditions is higher than with ordinary food. With the latter they reach 30 days only occasionally; the highest age recorded as yet with the altered diet was 42 days, that animal surviving weaning for 17 days.

This prolongation of the grey-lethal's life was to be expected since they are now independent of the declining lactation of the mother. Whether and to what extent developmental processes go on during this period remains still to be investigated. A general objection which can be raised against this experiment is that there is no control of the amount of food that each individual has actually eaten. No quantitative conclusions can therefore be drawn till this variable has been brought under experimental control.

We may conclude that the most important factor which determines the abnormal growth-curve is endogenous. Under favourable experimental conditions, however, life without any appreciable increase of weight can be prolonged for some time. To what extent this is possible with improved technique remains to be shown by further experiments.

4—Structure of Teeth and Bones of the Grey-Lethal

(*a*) *X-ray Technique*—In order to find out whether there is absence of the anlage for the teeth or just a retention, X-ray photographs were taken.

Figs. 13 and 14, Plate 19, show the halved heads of a normal and a grey-lethal of 28 days in side-view. Teeth are present in the grey-lethal, but they are very abnormal. The upper incisor is a little hook instead of a large segment of a circle. The lower incisor is a slightly curved and badly calcified small cone which can be traced back by this method only to the neighbourhood of the first molar. The molars which, particularly in the mandible, show nicely calcified roots in the normal show not a trace of roots in the grey-lethal.

Other differences are found in some of the skull bones. The zygomatic arch, the hyoid, and the neck vertebrae are much more heavily calcified in the grey-lethal than in the normal.

Fig. 15, Plate 19, shows two macerated skulls in top view. Evidently the calcification of the molars, particularly of the wisdom teeth, is incomplete in the grey-lethal as compared with the normal, On the other hand, the pterygoid processes show up more prominently in the grey-

lethal. The most striking difference is to be found in the turbinal bones, which are very heavily calcified in the grey-lethal but hardly visible at all in the normal. Other obvious differences in shape of the skull will be dealt with later in this paper.

Fig. 19, Plate 20, shows the complete bodies of a normal litter mate and a grey-lethal of 27 days. Very pronounced differences are found in the long bones, as humerus, femur, and tibia. Generally, calcification is very much heavier in the grey-lethal, particularly in the shafts in the vicinity of the epiphyses; on the other hand, the outline of these bones is less well defined, the edge being more or less diffuse, while in the normal there are very sharp outlines. In contrast to this increased ossification, bones which are already calcified in the normal are not yet noticeably calcified in the grey-lethal. This applies, for example, to the head of the femur, several bones in the hands and feet, and also the vertebrae, particularly of the tail, are very much behind.

X-ray photographs were taken in young animals and were repeated every 3rd or 4th day to see the time-relations in calcification. No difference was found in new-born animals. On the 3rd day a slight but clearly noticeable difference is visible. On the 7th day it is already quite pronounced, fig. 18, Plate 20; it becomes more and more intense the older the animals grow. In general calcification seems to lag behind in the grey-lethals; this is particularly noticeable for the epiphyses of the long bones and for the tail vertebrae. Once calcification has started the bones become more dense than in the normal.

(*b*) *Dissection*—On dissection of young animals a marked difference is found in the surface of most of the bones (excluding the flat membrane bones of the neurocranium). The surface of normal bones is practically smooth and shows comparatively little structure. In the grey-lethal the surface is more or less unfinished, showing shallow grooves and holes. Some of the spongiosa is usually visible. Bone sutures, *e.g.*, on the base of the skull, are always less distinct than in normals of the same age.

The teeth in both jaws are found to lie in their sockets and do not show any immediate signs of eruption. The upper incisors occasionally behave somewhat differently; their very tips may be visible just at the level of the mucous membrane, but continued observation shows that they do not erupt fully. This behaviour is not surprising since the tips of the mouse incisors are always very near the surface of the mouth epithelium and are never in development covered by the bone of the sockets.

The shape of the crowns of the molars shows no appreciable difference from normal molars; only the lower first molars are somewhat more

compressed in the bucco-lingual direction. The roots of the molars are usually more or less developed, but they are soft and flexible, being obviously uncalcified. The dorsal surface of the maxilla usually shows very wide nerve foramina through which the molar root-tips are often seen to protrude somewhat into the orbit.

In the mandible the most striking feature is a very large and deep mental foramen, which is almost exactly circular with a sharp edge and steep walls. It increases in size with age and in older specimens exceeds that of a normal sib in diameter by roughly 10–15 times. Unlike that of a normal animal, it is not pierced by the mental nerve and artery alone; on the contrary, it is sometimes difficult to find these structures at all when dissecting under the binocular. The contents of the large cavity consist of a bulge of dense tissue which in older specimens protrudes considerably above the surface of the mandible, like the cork of a champagne bottle, and is only covered by the periosteum of the mandible. In older specimens it is always palpable and often even visible *in vivo*. This node of tissue is more or less globular and can easily be separated from the surrounding structures. A careful dissection of this isolated piece shows that besides soft tissues it contains some hard sheets of calcified matter which, according to their consistency, colour, and surface structure look like parts of teeth. The shape of these structures is very variable and often quite different on both sides of the same mandible. The variability is so great that it is hardly possible to find common characteristics which fit all of them. Usually the thin sheets of calcified matter have one concave and one convex surface, like the valves of a lamellibranch. Sometimes the whole structure is more or less tubular with a cavity inside; such a tube may have more or less bulging protrusions of sub-globular or ovoid shape; the outer end may or may not be closed. Occasionally two independent calcified sheets are found, one of which more or less includes the other and is only separated from it by a soft, somewhat gelatinous tissue like tooth pulp. The variability of these structures is thus very great, indicating that they are formed under quite different mechanical conditions which obviously are not controlled in a proper way in development. It will be seen later that this peculiar structure is developed from the back end of the lower incisor.

The lower incisor is a rather poorly calcified thin-walled tube of slightly curved conical form. It is best calcified near the tip; its density decreases gradually backwards so that it is hardly possible by dissection to determine the position of the back end; it can be traced back to the neighbourhood of the front end of the first molar.

Calcification is much stronger in the upper incisor which forms a short,

strong element in outline not unlike to the bill of an eagle. The small hook at its tip corresponds to that found in all normal upper incisors just at cutting which is soon worn off by use. The backward limit of calcification is much more sudden, but some uncalcified matter is always attached to the rear end.

(*c*) *Histology*—The histological examination of the bones reveals striking abnormalities. Fig. 4 shows the tibiae of a grey-lethal and a normal litter mate of 23 days. In the normal, some spongiosa is present near the epiphysis, but towards the shaft of the bone no spongiosa is left at all, the whole cavity being filled up by bone marrow. The shaft consists entirely of strong perichondral bone. In the grey-lethal tibia the spongiosa extends through the whole shaft; although no large common cavity for the bone marrow is formed, the density of the spongiosa is somewhat lessened in the middle parts of the shaft. On the other hand, the development of the perichondral bone is very poor, although it is never quite absent. The whole bone structure thus somewhat resembles that regularly found in Cetacea.

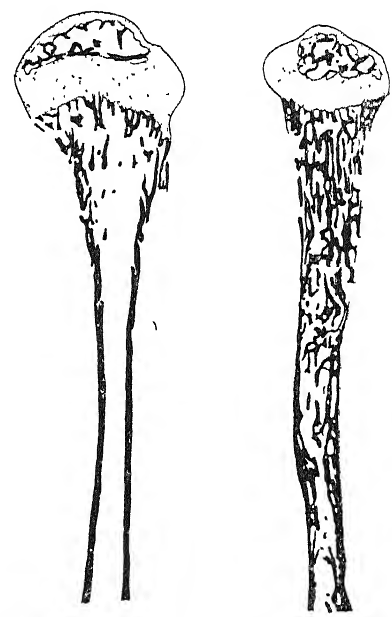


Fig. 4—Tibiae of a grey-lethal and a normal litter mate (left), 23 days old. Bone black, cartilage hatched

These histological differences explain those found in the X-ray photographs and by dissection. The density of the shadows in the X-ray photographs of the grey-lethal is caused by the enormous amount of spongiosa, while the diffuse outlines of the bone are explained by the poor development of the periosteal bone. The latter fact finds another expression in the unfinished appearance of bone surface at dissection.

A further difference regularly observed is the fact that many spicules of the spongiosa are covered with or consist of uncalcified bone ("osteoid") to an extent not met with in normal animals.

The anomalies of the bones can therefore be described as—

(1) persistence of all the spongiosa once it is formed;
(2) poor development of perichondral bone;
(3) incomplete calcification of the spongiosa spicules.

Such anomalies have been found in all the bones so far investigated, with the exception of the flat membrane bones of the neurocranium, which have a normal appearance.

Fig. 5 represents two corresponding sections through the basi-sphenoid which need no further explanation. It may be pointed out that this type of anomaly is not confined to cartilage bones, but is found similarly in membrane bones, as is to be seen, *e.g.*, in figs. 20 and 21, Plate 21, for the mandible.

Whether the persistence of all the spongiosa is caused by a reduction of the osteoclasts in number or by a decreased activity of these cells is a question which remains to be investigated.

The teeth were investigated in ground sections and in decalcified series. Very little if any differences were found in the structure of the enamel.

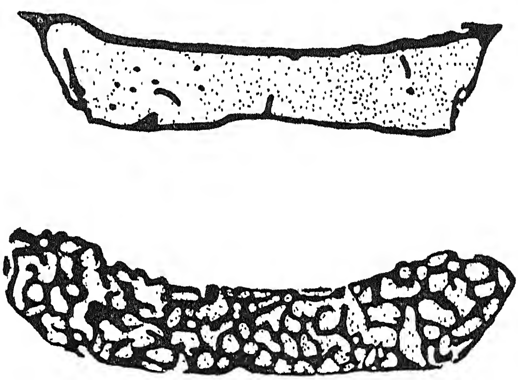


FIG. 5—Transverse sections through the basi-sphenoid of a normal (18 days, above) and a grey-lethal (20 days). Bone black, bone marrow hatched

Figs. 13 and 14, Plate 19, represent the upper incisors of a normal and of a grey-lethal. In the grey-lethal it can be seen that the original tip consisting of osteodentine is preserved.

In decalcified sections the most striking difference found in the dentine is the presence of a very wide layer of uncalcified matter (" dentinoid ") in all the grey-lethal teeth, while in normals of the same age this layer is very much thinner. While the calcification of the dentine extends down to the very tips of the roots in the normal, only parts of the crown dentine are calcified in the grey-lethal. This is clearly seen in figs. 22–25, Plate 21, for different molars and also applies to the incisors. On the other hand, the walls of the roots are thicker in the grey-lethal.

It can therefore be stated that there is in the teeth a lack of co-ordination between dentine formation and dentine calcification; while calci-

fication stops, dentine formation continues. As a result the root tips remain uncalcified and flexible and therefore do not cast shadows in the X-ray photographs. It is obvious that there we have got a close parallel to what is happening in the bones, where the later-formed layers of spongiosa spicules are uncalcified.

The further development of the roots of the teeth is determined by the interaction of tooth and bone anomalies. When the soft roots are growing against the abnormally dense structure of the spongiosa they become bent and wrinkled in a most complicated way. One sees quite clearly how the roots are plastically pushed and pressed into the smaller cavities left by the spongiosa spicules, and how the particular form of bending is obviously determined by the resistance the growing root finds in the spongiosa. This is clearly visible in fig. 22, Plate 21. This type is realized in all the lower molars and in the upper incisors.

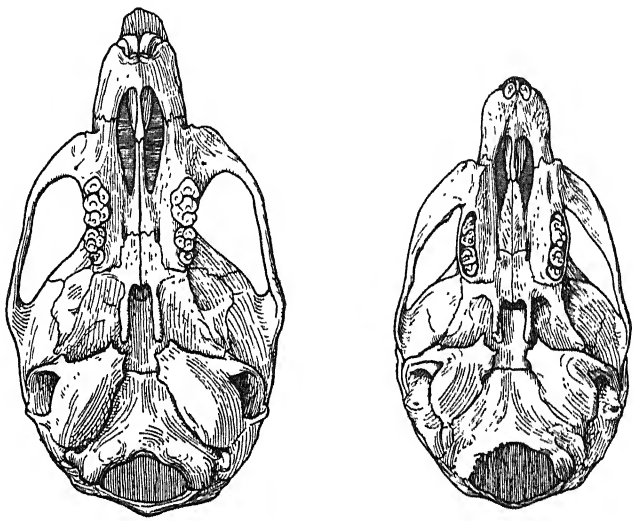
The anatomical situation is quite different for the upper molars. In the normal the roots are covered only by a layer of periosteal bone. Since in the grey-lethal this component of the bone is very poorly developed and moreover amply perforated by the branches of nerves and arteries leading through that layer to the teeth, the roots make use of these pre-established holes and grow backwards into the orbit, as described in the preceding section. Since the growth tendency of molar roots is limited as compared with that of the incisors, this does not lead here to very excessive structures. The backward growth of the roots of the upper molars in the grey-lethal is, however, fairly constant and is, at least at the age of about three weeks, found in almost every root of each specimen. Occasionally, a root does not find its proper foramen; in this case, similarly bent roots are formed as are always found in the lower molars. Molar roots leaving the maxilla dorsally are shown in fig. 24, Plate 21.

The most striking difference is found in the lower incisor. Here a combination of the two types just described occurs. The rear end leaves the mandible through the mental foramen and forms here the large bulge described in the previous section. Since the growth tendency of this tooth is not limited as in the molars it is bound to get bent and folded, although it has found the way out, since even here the space available is limited. What is peculiar to this outgrowing back end is the fact that here calcification starts independently; therefore the remarkably variable sheets of calcified matter are formed which were described above. Another centre for calcification is found in the vicinity of the tooth tip, but both centres are separated by uncalcified parts, which again are folded in a very complicated way. The fact that this uncalcified and very delicate intermediate section is the only connection between the two calcification

centres, is the reason why it was not found in the first dissections that both structures were parts of one and the same tooth. Figs. 20 and 21, Plate 21, show this situation quite clearly. In fig. 21 just the connecting string of tooth pulp with odontoblasts and adamantoblasts is cut in the section.

5—Shape of Skull, Mandible, and Skeleton of the Grey-lethal

The striking differences constantly found in the shape of the skull are well shown in fig. 15, Plate 19, and in fig. 6. First of all, the whole snout



a b

FIG. 6—Skulls of a grey-lethal and a normal (left) of 30 days. Same specimens as in fig. 15, Plate 19

is considerably shortened in the grey-lethal while the size of the neurocranium is little if at all affected. There is little doubt that this is due to the fact that the stimulus of the growing upper incisor is very much reduced.

Furthermore, the shape of the zygomatic arch is quite different. In the normal it is much wider, particularly in its frontal parts, which are broadly curved outwards, while in the grey-lethal there is a very marked acute angle. The zygoma is important as being the point of origin for the masseter; to the median side of it, other masticatory muscles are situated, such as parts of the temporalis and the inner portion of the masseter which in rodents originates from the inner surface of the infraorbital foramen. Similarly the pterygoid processes which serve as origin

for the internal pterygoid are reduced in size. Since the whole method of feeding is fundamentally altered in the grey-lethals which never make that use of their teeth which has given the name to the order of rodents, it is reasonable to assume that these abnormalities of shape are causally connected with the altered or rather reduced use of the masticatory muscles. Similar evidence comes from the mandibles of fig. 7. The corpus of the mandible as a whole is reduced and forms a somewhat more obtuse angle with the ramus than in the normal; the whole proportions are somewhat embryonic. The condyloid process, and particularly the joint surface, is very poorly developed in the grey-lethal as compared with a normal. The difference in the mental foramen has been dealt with in a previous section. Other differences are easily seen from the

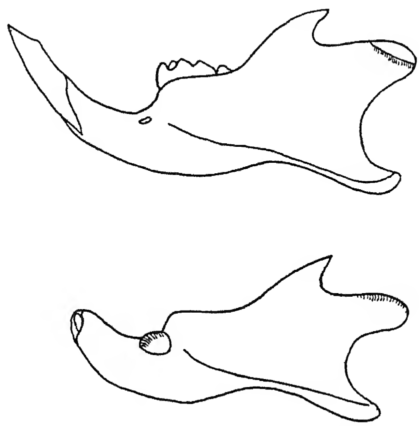


FIG. 7—Mandibles of a grey-lethal and a normal litter mate (above). 28 days old. The extra-mandibular part of the lower incisor removed from the mental foramen in the grey-lethal

drawings. We do not intend to go into any more details here, since the better evaluation of these secondary or tertiary effects of altered feeding habits on skull and mandible shape needs a more thorough investigation.

One more drawing may be given here which shows that relation rather neatly. Fig. 8 shows the corresponding* sections through the infraorbital foramen. In the grey-lethal the foramen is clearly more compressed laterally, and correspondingly the cross-section of the inner portion of the masseter is considerably smaller. Obviously the narrowness of the foramen is caused by the relative inactivity of the muscle.

* Sections strictly corresponding for all the organs cannot be found in such a series since the whole proportions of the heads are altered; in this case correspondence is maintained by the fact that the foramen is cut as a real foramen surrounded everywhere by bone.

The underdevelopment of the masseter is obviously caused by the lack of cut teeth, which in turn is caused by the lack of co-ordination of dentine formation and dentine calcification and so on.

Another difference is the narrowness of the rear parts of the nasal duct in the dorso-ventral direction.

While some of the differences in skull and mandible shape are to be explained by the changed feeding habits another group of differences seems to depend on other causes.

Fig. 9 shows two corresponding sections through the nasal region just in front of the anterior end of the cleft of the palate. Apart from other differences in bone structure already dealt with in a previous chapter,

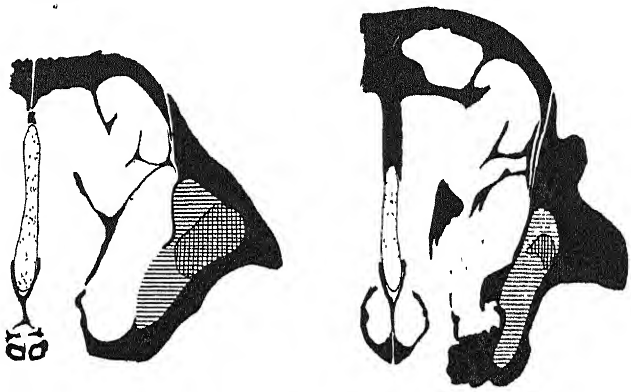


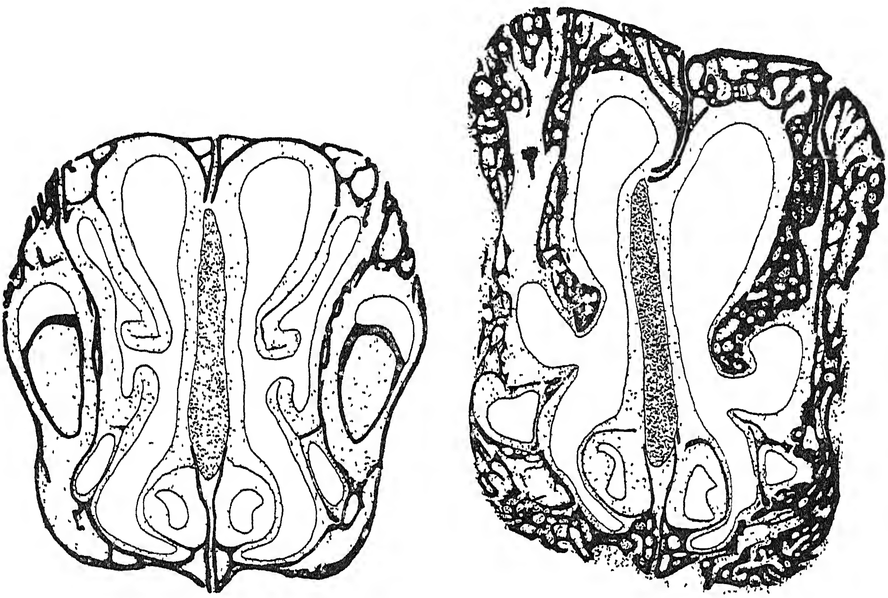
FIG. 8—Transverse sections through the infraorbital foramen of a grey-lethal (20 days) and a normal (18 days, left). Parallel lines indicate the infraorbital foramen, crossed lines the section through the inner portion of the masseter

there is an excessive development of the upper (and, to a lesser extent, of the lower) turbinal bone in the grey-lethal as compared with the normal. Instead of being a very thin sheet of bone there is an enormous spongy mass which to some extent is liable to alter the whole conditions of the air passage. This and similar excessive bone formation is responsible for the appearance of the unusual shadows in the nasal region in the X-ray photograph, fig. 15, Plate 19.

Similar excessive bone formations cause differences in the bone relief in the palatine region, as shown in cross-sections in fig. 9 and in top view in fig. 6 (above). Note particularly the differences in shape of the palatinal cleft which in the grey-lethal is narrowed in the middle by an accessory bony plate of a narrow triangular shape originating from the vomer.

Another anomaly seen in the cross-sections, fig. 9, seems also not to be accidental. The nasal septum of the grey-lethal is quite asymmetrical;

it is obviously pressed into that abnormal position by the excessive growth of the vertical process of the nasal bones. Here apparently the lack of co-ordination of the growth of the cartilage septum and the process of the nasal bones leads to an anomaly which is essentially the same as the septal deviations so often found in man. Exactly the same condition, corresponding in all details including the direction of asymmetry, has been found in both grey-lethal heads of which complete series of sections were made. It can thus hardly be regarded as accidental.



a *b*

FIG. 9—Transverse sections through nasal region just in front of anterior end of cleft in palate. Left, normal; right, grey-lethal. Same specimens as in figs. 5 and 8

Another phenomenon already mentioned may find its explanation in the altered shape of the nasal region. The eyes of the grey-lethals are regularly found to be more or less closed, the eyelids sticking together to an extent which varies from day to day. Close inspection of the cornea and conjunctiva reveals no clinical symptoms of inflammatory processes. In sections the lacrimal duct is filled with epithelial cells, but no leucocytes are present; the phenomenon is thus due to some moulting of the epithelium in the lacrimal passages. Actually, the same cells, but in much smaller numbers, are found in the controls. The difference is probably due to the fact that in the short nose of the grey-lethal the lacrimal duct is more sharply bent on its way down to the nasal duct,

thus rendering the passage of the lacrimal fluid with the epithelial cells more difficult. No complete stenosis of the duct could be found in complete series of sections; the existence of a complete stenosis was, however, unlikely since the varying conditions of the eyes show that the animals are not absolutely unable to get rid of that detritus.

Further differences in shape are found in most of the limb bones, the shoulder girdle, and the pelvis. Fig. 10 shows the femur of a grey-lethal and a normal litter mate of 28 days. For comparison, a femur of a younger normal (13 days old) corresponding in linear measurements to the grey-lethal is added. The grey-lethal bone is much shorter and at its distal end much clumsier than that of the sib, but corresponds fairly well

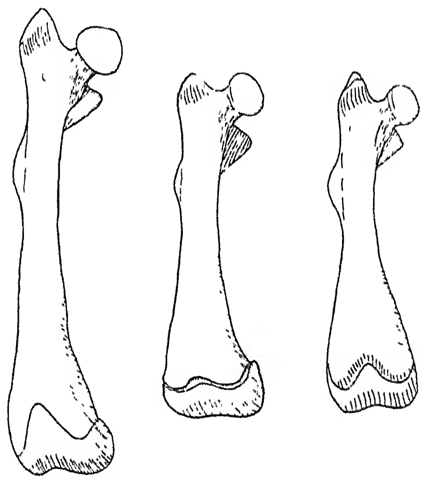


FIG. 10—Femurs of grey-lethal (right) and normal litter mate (left) of 28 days. For comparison femur of 13-days-old normal in the middle

to the younger normal. It seems, therefore, that difference in size is largely due to an arrest in development, the grey-lethal exhibiting that stage in bone development permanently which was reached at the turning-point of its growth-curve at the age of about a fortnight.

This type of difference, however, is not the only one found in the bones.. Fig. 11 shows the humeri of the same three specimens. Here again the grey-lethal bone corresponds roughly to the younger stage in its general proportions. The proximal end of the bone, however, is much clumsier, the head projects much less, and there is a quite different shape of the deltoid ridge. All these anomalies are not to be explained in terms of arrested development only, but must find their explanation in the anomalous mode of bone growth.

The same situation is illustrated by the tibiae and fibulae of fig. 12.

Here again the incomplete fusion of tibia and fibula in the grey-lethal corresponds fairly well to the condition found in the 13-days-old normal. But instead of the elegantly curved form with its pronounced surface relief we find a bone with very clumsy proportions. The crista anterior tibiae and the tuberositas tibiae are very rudimentary, the facies lateralis tibiae is convex instead of concave, the crista interossea is a wide, smoothly rounded prominence instead of a sharp crest. Similarly the shape of the fibula is altered. The bones look somewhat as if a wax model of normal bones had been slightly warmed so that the characteristic shape had been replaced by more rounded outlines.

Similar differences were found in the scapula, radius, ulna, and in the pelvis.

[*Note added in proof July* 29, 1935.—The condition found in the bones of the limbs leaves no doubt that there is failure of the secondary modelling of the bone surface by absorption. This is clearly seen by the fact that it is always the growing ends of the bones which show the pronounced anomalies, viz., the proximal end of humerus and tibia, the distal end of femur and radius. Evidently this is completely analogous to the failure of resorption of the spongiosa described above, and it accounts, too, for the excessive development of the turbinals, the narrowness of the cleft of the palate, and most probably for the anomalous shape of the zygomatic arch.]

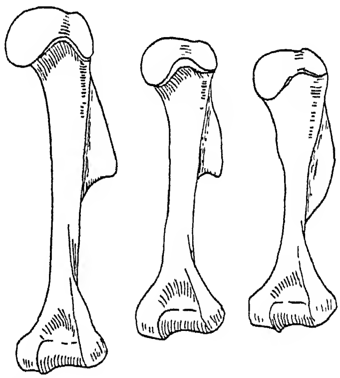


FIG. 11 — Humeri of the same three animals as in fig. 10

Thus we may state that the differences in shape of skull, mandible, and skeleton seem to be caused in at least four different ways.

(1) Shortening of the snout due to deficient stimulus from growing upper incisor.

(2) Alterations of those parts which are affected by the reduced action of the masticatory muscles (*e.g.*, shape of infraorbital foramen).

(3) General arrest of development.

(4) Anomalies due to the absence of remodelling processes of the bone surface (*e.g.*, shape of the growing ends of the long bones).

This scheme is of a preliminary character, and particularly on skull and mandible it is sometimes difficult to decide to what extent the altered feeding habits or the lack of secondary bone absorption is responsible for differences in bone shape.

It may be mentioned in passing that the general arrest of bone-growth is already noticeable in the cartilage. In the proximal epiphysis cartilage of the tibiae, drawn in fig. 4, numerous mitotic divisions are found in the columnar layer in the normal, while none is present in the grey-lethal. Correspondingly, the width of the columnar layer is here reduced.

6—Discussion

We have to deal apparently with a single Mendelian factor which causes disturbances in a variety of organs and a whole hierarchy of secondary and tertiary effects. When we speak of primary causes we must recognize that the primary mode of action of this gene is far from being discovered. For example, no common origin has been found for the bone and tooth abnormalities on the one hand and the coat colour on the other. The skeletal changes are expressed in a variety of ways which have not yet been fully analysed.

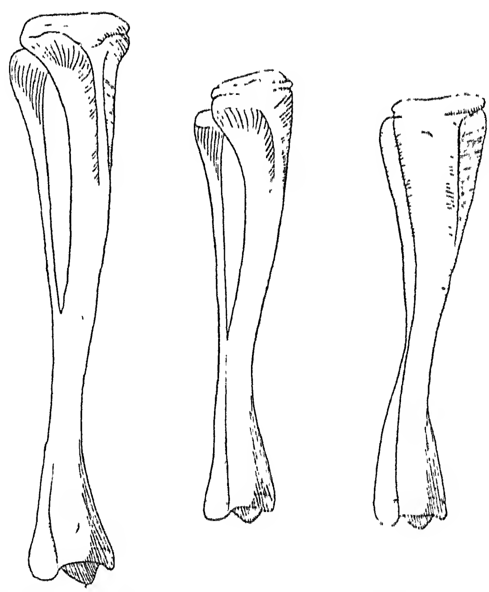


Fig. 12—Tibiae and fibulae of the same three animals as in figs. 10 and 11

A detailed study of all the inner organs is being made, especially of the endocrine glands. This is to be supplemented by physiological experiments and it is hoped in this way to locate the root cause. The author is aware of no pathological condition in man or mammals which can be closely compared with the complex of symptoms in the grey-lethal. For

the present it remains uncertain whether the ultimate cause is an endocrine disturbance, an alteration in metabolism, or some other factor. At the present stage of the work such speculations are premature.

The author is indebted to Professor J. B. S. Haldane, F.R.S., Professor D. M. S. Watson, F.R.S., and to Mr. J. T. Carter for several valuable hints and suggestions. Mr. F. Melville (X-ray Department, University College, London) did the X-ray photographs, Mr. C. H. Osterstock (John Innes Horticultural Institution, Merton) took the microphotographs, and Miss J. Townend (Zoology Department, U.C.L.) drew fig. 6 for this paper. Mr. F. C. Minns kindly read through the manuscript. My particular thanks are due to our technical assistant, Miss E. A. Milsom, who devoted much care to the feeding experiments. The work was done whilst the author was in receipt of a grant from the Academic Committee of the Central British Fund for German Jewry which he gratefully acknowledges.

7—Summary

An autosomal recessive gene, called grey-lethal, is described in the house mouse which suppresses the formation of all the yellow pigment in the coat and kills the homozygotes at the age of 22–30 days.

Growth-curves show at first an increase followed by a period of weight-constancy and eventually weight-loss and death; taking the mother away from the litter at normal weaning age leads to rapid death of the grey-lethals. A supply of finely divided food prolongs the life of such animals up to 42 days, but the animals seem not to continue their general development during that time, but only to maintain the *status quo*.

Investigation of teeth and bones by means of X-rays, dissection, and histological preparations revealed a lack of co-ordination of dentine formation and dentine calcification leading to a complete retention of all the teeth; in the bones, persistence of all spongiosa spicules, poor development of periosteal bone, and incomplete calcification are found in all the cartilage and membrane bones with the exception of the flat bones of the neurocranium.

The shape of the grey-lethal skull, mandible, and skeleton is altered by lack of growth stimulus as caused by the rudimentary upper incisor, by reduced use of the masticatory muscles, by lack of secondary absorption remodelling the bone surface, and by a general arrest of bone-growth.

Explanation of Plates

Plate 19

Fig. 13—Halved head of normal, 28 days old. X-ray photograph. × 2.

Fig. 14—Halved head of grey-lethal, 26 days old. X-ray photograph. × 2.

Fig. 15—Macerated skulls of normal (left) and grey-lethal (30 days old). X-ray photograph. × 2.

Fig. 16—Right upper incisor of normal, 29 days old. Unstained ground section. × 22·5.

Fig. 17—Left upper incisor of grey-lethal, 29 days old. Unstained ground section. × 22·5.

Plate 20

Fig. 18—Grey-lethal (right) and normal litter mate (left), 7 days old. X-ray photograph.

Fig. 19—Grey-lethal (right) and normal litter mate (left), 27 days old. X-ray photograph.

Plate 21

Fig. 20—Transverse section through grey-lethal mandible (19 days old), just in front of the mental foramen. Left lower incisor in the centre, its extra-mandibular portion on the right. Suza fixation, haematoxylin-eosin, 15 μ. × 39.

Fig. 21—Transverse section through the mental foramen of same mandible showing the connection between the intra-mandibular and the extra-mandibular parts of the lower incisor. Same technique.

Fig. 22—Left lower first molar of same grey-lethal. Same technique.

Fig. 23—Right lower first molar of normal (18 days). Formalin, haematoxylin-eosin, 15 μ. × 39.

Fig. 24—Right upper second molar of grey-lethal. Same animal as in figs. 20–22.

Fig. 25—Left upper second molar of normal. Same animal as in fig. 23.

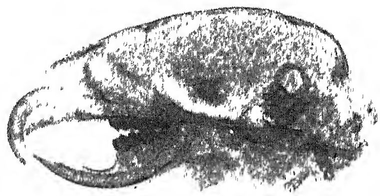


FIG. 13

FIG. 14

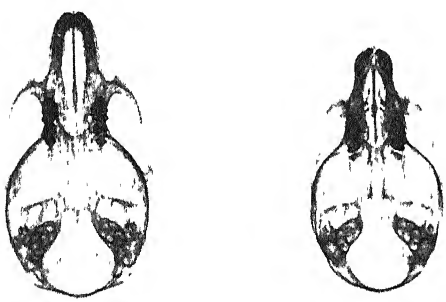


FIG. 15

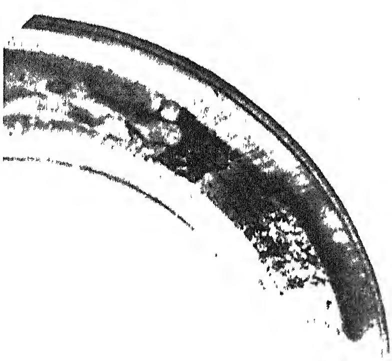


FIG. 16

FIG. 17

(*Facing p.* 342)

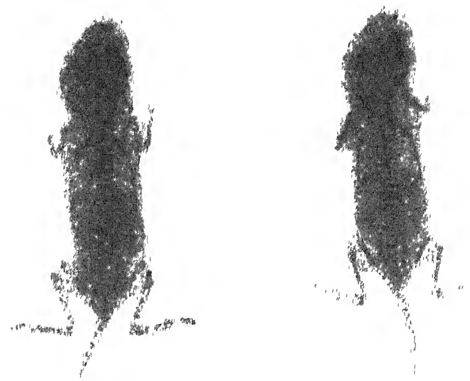


FIG. 18

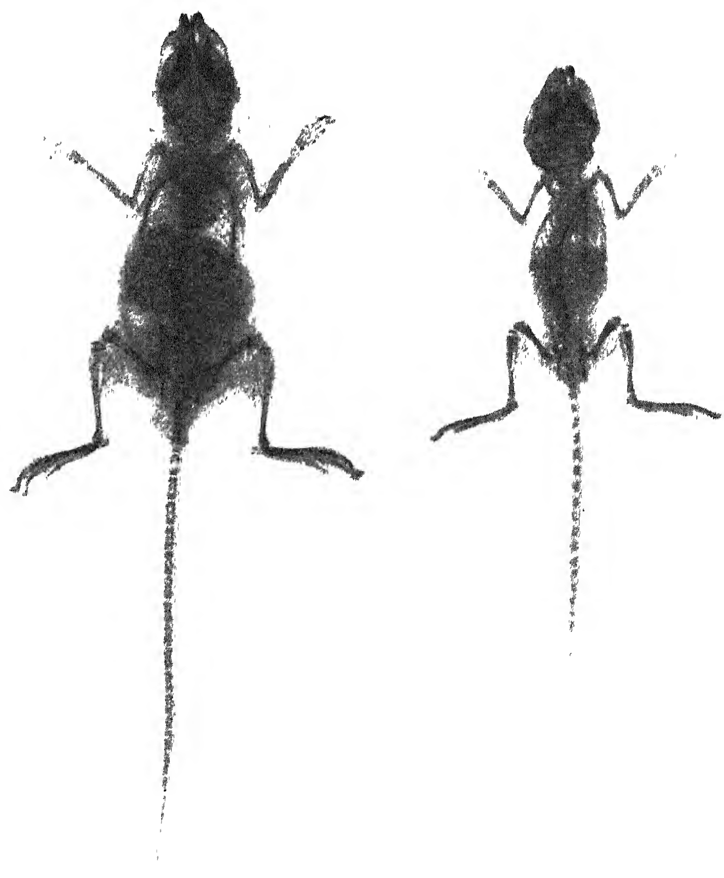


FIG. 19

FIG. 20

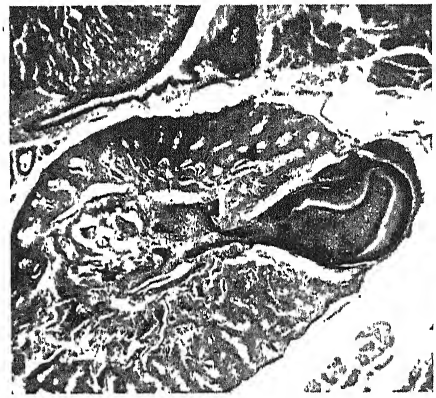


FIG. 21

FIG. 22

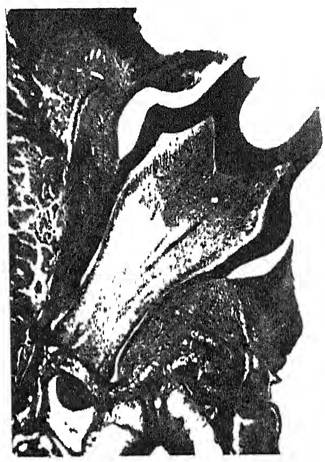


FIG. 23

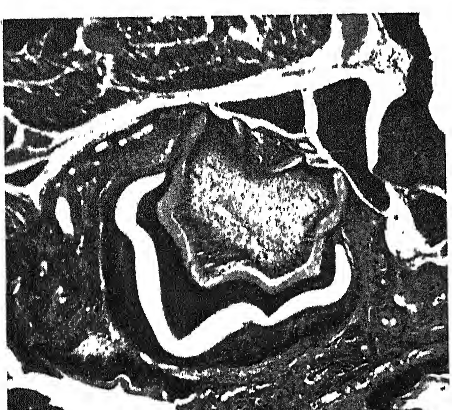


FIG. 24

FIG. 25

633 . 51 : 551 . 577

Rainfall and Cotton Yields in the Sudan Gezira

By EDWARD M. CROWTHER, D.Sc., F.I.C., Chemistry Department, Rothamsted Experimental Station, Harpenden, Herts, and FRANK CROWTHER, D.Sc., formerly Plant Physiologist, Gezira Agricultural Research Service, Sudan Government.

(*Communicated by Sir John Russell, F.R.S.—Received June* 3, 1935)

INTRODUCTION

Agricultural meteorology makes slow progress because it is rarely possible to study the correlation of weather and yield for crops grown in normal agricultural rotations over long periods. Both in commercial agriculture and at experiment stations, rotations and cultural methods change sufficiently frequently to destroy the homogeneity of the data, even when records are kept for specific fields or farms. In some of the newer agricultural areas, and especially in those under irrigation, the cropping is simpler, and the need for records and field experiments is more keenly appreciated. Research programmes and practical measures must be determined from experience over only a few decades; but erroneous conclusions may easily be drawn from trends over such short periods, memories of individual years, or simple correlations of yields and single weather variates. In the present paper, modern statistical methods of analysing small samples of data are used to examine the relationships between rainfall and cotton yields under irrigation in the Sudan Gezira for periods up to 23 years.

The mean yields from this large and semi-desert area fluctuate violently from year to year in such a way as to suggest the dominance of some climatic factor. It will be shown that the cotton yields are highly correlated with rainfall, and further, that seasonal fluctuations in rainfall, including certain cyclic changes, are sufficient to account for most of the decline in yield on some of the oldest areas. This conclusion has considerable practical importance, because a recent sequence of low yields led some critics (Balls, 1935, Vageler and Alten, 1932) of the scheme to conclude that irrigation without drainage had already produced serious soil deterioration. The significance of some of the rainfall effects established may direct attention to new methods of studying problems of soil fertility and the control of pests.

Earlier Analyses

In 1925, E. M. Crowther (1926) found that the yields of cotton grown under irrigation in the normal three-course rotation of the Sudan Gezira appeared to be negatively correlated with the amount of rain in May and June, even though the greater part of the season's rainfall was in July and August, and the cotton was not sown until August. The evidence was admittedly scanty, but it included all the data then available for cotton grown under uniform crop rotations. At the time the suggested importance of early rains aroused little interest among the local workers, but more recently a run of several low yields in years with high early rains, broken by a very good year with unusually late rains, led to an exaggerated confidence in the importance of the effect, and to numerous *ad hoc* hypotheses to explain it.

In 1931 Hewison, who as Director of Agriculture in the Sudan Government was associated with the Gezira scheme from its inception, published a graphical analysis of the yields and total rainfall for the oldest centre—Taiyiba—from 1911 to 1931. He ignored the effects of early rains, but concluded that high total rainfall was a primary cause of low seasonal yields, and suggested some of the ways in which heavy rains might interfere with the cultivation and growth of the crop. Hewison also noticed an apparent increase in the total annual rainfall at Taiyiba over the period, without realizing, however, that this circumstance weakened the evidence for his conclusion. If irrigation caused some progressive deterioration of the soil or increase in weeds and pests during a period in which rainfall also increased, yields and rainfall would necessarily be correlated. In the period from 1911 to 1931 the yield was above the average in seven of the first nine years, and rainfall was below the average in eight of these nine years. It is not possible, therefore, to decide whether the lower yields in the second half of the period were due to extra rainfall or to some other slow change in soil or environmental conditions. Clearly, progressive changes and annual fluctuations should be separated before testing correlations which it is hoped may reveal causal relationships. Hewison also noted that spells of a few wet or dry years tended to occur together.

In 1933 F. Crowther showed that the Taiyiba yields gave negative partial regressions on both early and total rainfall. In other words, atlhough rainfall as a whole was harmful, a given quantity of rain had a more deleterious effect early in the season than later on.

THE SUDAN GEZIRA

The Sudan Gezira, fig. 1, may be defined as the roughly triangular area bounded by the Blue and the White Niles and the railway from the

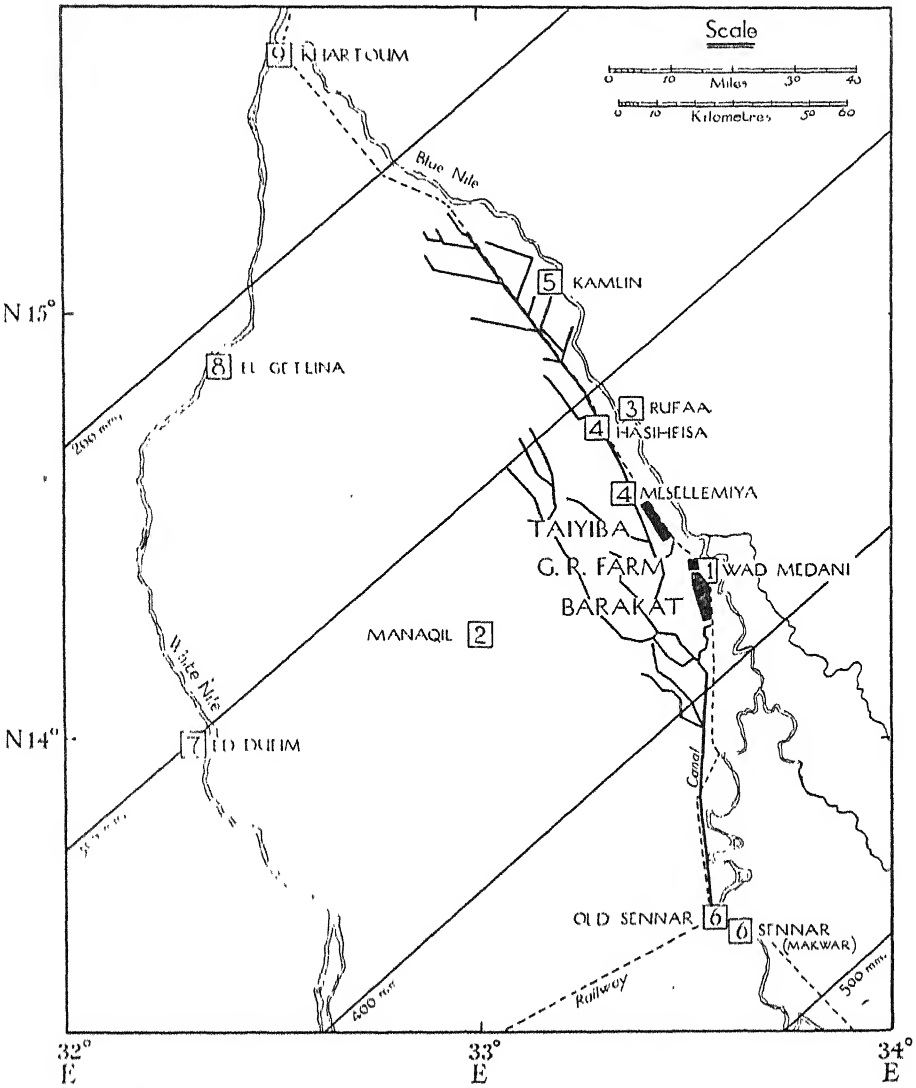


FIG. 1—Map of Sudan Gezira showing the earliest cotton areas, Taiyiba, Barakat, and the Gezira Research Farm the present canalization, the position of nine rain gauges, and the calculated isohyetes for mean annual rainfall

Sennar dam to Kosti. Cotton has been grown in the Eastern Gezira under irrigation since 1911, the water being lifted from the Blue Nile by pumps until 1925, and by the Sennar dam subsequently. Over most of

the irrigated area the soil is a deep, heavy, alkaline clay overlying a deep but less heavy alluvial deposit. Little of the irrigation water penetrates beyond one metre, below which depth accumulations of sodium and calcium salts are common. The soil was fully described, with analytical data, by Greene (1928, *a*, *b*) and discussed more generally by Vageler and F. Alten (1932). Throughout much of the season the cotton is liable to show temporary wilting of the leaves under the intense radiation and extremely low atmospheric humidity, but, in spite of this, all of the significant correlations established in this paper show that extra rainfall at any period is harmful.

Before irrigation was introduced, much of the land was heavily cropped with dura (*Sorghum vulgare* or *caudatum*, millet), rain-water being accumulated by means of light earth banks. A pump station for cotton irrigation was opened at Taiyiba in 1911, a second one at Barakat in 1914, and a few others from 1921 to 1924 to facilitate the change to large-scale irrigation in 1925. The Gezira Research Farm was set up in 1918 at the north of the Barakat Farm.

The analysis of yield records for these centres is complicated by the circumstance that, as the pioneers gained experience, they frequently brought in new land and changed the crop rotations and methods of cultivation without retaining experimental record areas under uniform treatment. The actual yields for individual tenancies (generally with 10 acres of cotton annually) were always carefully recorded and mapped, since they formed the basis of payment to the native cultivator. Through the courtesy of the Sudan Plantations Syndicate, Ltd., we have had access to these records from the first cultivation of the two oldest farms up to the 1931–32 season, and also to their rainfall records and confidential monthly reports. We have thus been able to prepare our data from the original sources, and to select areas and periods in which the cultural treatments of the crop were most nearly uniform. The mean yields from some of these areas are given in Table II, as they are not accessible elsewhere. Some detailed accounts of the areas selected for analysis will be given later, but at this stage it will be sufficient to outline the customary cultivation of the crop from about 1918 to 1931. Cotton was grown every third year, and was generally followed by either dura or lubia (*Dolichos Lablab*), grown with summer rains and minimal irrigations, and then by a year's fallow during which the land was cultivated by heavy power tackle and set up in ridges. Rainstorms increase in frequency and amount from occasional showers in May and June to a maximum in August. Rains are slight in September and October and rare later. As the cotton is sown about the middle of August after an irrigation, heavy rains in this month delay

sowing, and may necessitate one or more resowings. Fortnightly irrigations are given from October to March.

Two of the more obvious causes of low seasonal yields are a bacterial disease Blackarm* --and a virus disease--Leaf Curl. The important secondary infection by Blackarm is distributed from infected foci by the splash of rain. The virus disease, which became serious about 1928, is distributed by white fly from ratoon cotton and certain weeds. Formerly the plants were cut off at ground level for burning at the end of the picking season in April, but recently the plants with their tap roots have been pulled out to reduce the risk of a redistribution of diseases from ratoon cotton in the following season.

THE METHOD OF ANALYSIS

Since the yield data are limited to the period 1911 to 1933, it is impossible to secure independent groups of seasonal data ; separate parts of this run of years would be too short for satisfactory estimation of the errors of the regression equations. The only alternative is to take, first, the longest set of years and the largest areas, and, second, periods in which the agricultural factors and the actual soils were substantially constant. Although the different selections must be correlated by including many years in common, we have always used the whole of the relevant data available. It is, of course, important to avoid the spuriously high correlations which might be obtained by picking out a few selected weather variates found by trial and error to give promising results for the individual groups of data. Some high chance correlations would probably be found during the short period for which the Gezira has had a first-class meteorological equipment, provided a sufficient number of determined trials was made for different weather variates. Apart from the Gezira Research Farm, the meteorological data have until recently been restricted to rainfalls at the two oldest cotton centres and nine other places in the Gezira.

In his classical analysis of "The Influence of Rainfall on the Yield of Wheat at Rothamsted," Fisher (1924) discussed the effects of paucity of crop data in the general problem of evaluating the effects of weather on crops. He laid down the following conditions for arriving at unprejudiced results.

"1. The meteorological variates to be employed must be chosen without reference to the actual crop record.

* *Bacterium malvacearum*, first called *Pseudomonas malvacearum* by E. F. Smith in 1901, but amended by him to *Bacterium malvacearum* in 1905.

2—If multiple variates are to be used, allowance must be made for the positive bias of R^2.

3—Relationships of a complicated character should be sought only when long series of crop data are available."

It would not, for example, be satisfactory to work on rainfalls before and after sowing, or to make an arbitrary selection of rainstorms with more than a prescribed rainfall. Again, it would be unwise to pick out for one group of yield data those weather variates which gave apparently significant effects and to use other weather variates for another group of yield data. The problem is not to discover which set of weather variates provides the most efficient fitting of specified yield data, but the more general one of estimating the average effect of rainfall at different periods of the year.

It was not possible to use Fisher's elegant method of correlating residuals after eliminating slow changes by fitting high-order polynomials, but the precaution was taken of eliminating the effects of progressive (linear) changes with time from all multiple regressions of yield on weather variates. The multiple regression equations always included a term for the number of the year (t), which eliminated these linear changes and also provided an estimate for the rate of change of yield with time for constant annual values of the rainfall variates considered.

Comparatively high standards of significance are required. Throughout the Tables, coefficients corresponding to P values of from 0·05 to 0·02 are shown by one asterisk, those from 0·02 to 0·01 by two, and those less than 0·01 by three.

The Rainfall Data

Since 1906 rain-gauge readings have been taken at the nine stations given in Table I and shown in fig. 1. Through the courtesy of the Director of the Egyptian Irrigation Department, Khartoum, we have had access to the Official Records to the end of 1933. The mean monthly rainfall for the nine stations over 28 years was :—

	April	May	June	July	August	September	October
mm ..	2	9	26	92	118	47	8

Since the annual rainfall is distributed fairly symmetrically about a date early in August, we have used as the simplest adequate expressions of the season's rainfall the total amounts in the following periods: *e*. early, before July 1; *m*. middle, July 1 to August 31; *l*. late, after August 31.

The total rainfall (p) in the year previous to sowing the cotton was also examined. All dates refer to the year in which the rain falls and the cotton is sown; the crop is picked early in the following year.

The rainfalls increase fairly regularly from N.W. to S.E. across the Gezira in the direction of the Abyssinian Mountains. The data* in the first part of Table I show how closely the 28-year means for early and total rainfall agree with values calculated from the regression of rainfall on latitude and longitude. Calculated lines for equal total rainfall are shown in fig. 1. It will be noticed from the regression coefficients that the relative rates of increase towards the south-east are greater for early and late rains than for mid-season rains.

Table I, *a*, also gives the standard deviation per annum of early and total rainfall for each of the nine stations. In spite of the range of mean rainfalls, the standard deviations for total rainfall are of similar order, about 100 mm, throughout the whole of the Gezira.

The general means and standard deviations per annum for rainfall in the three periods and the annual total are summarized separately for the whole of the nine stations, Table I, *c*, and for the first five central stations in or near the present irrigated area, Table I, *b*. The annual values for the means of these five central stations are given in Table II. The standard deviations of the means of five or nine stations, Table I, *b* and *c*, show the magnitude of seasonal variations for the area as a whole. The standard deviations per station measure the average variation at specified points, and are important in considering the scale of the effects of rainfall on yield, which are subsequently expressed as regression coefficients of cotton yield on unit amounts of extra rainfall. Relative to their mean values, early and late rainfalls are much more variable than mid-season or total rainfalls. In round values the standard deviations in the irrigated area may be taken as 30, 90, 50, and 100 mm per annum for early, middle, late, and total rainfall respectively. Finally, in an analysis of variance of rainfall for five or nine centres over 28 years, the mean effects of stations and of years may be eliminated and the interaction between years and stations used to give a standard deviation per station, which measures the variability of rainfall between the stations in a single year. Although the effects of year and position are highly significant, rainfall variations within the Gezira are still sufficiently large to make it desirable to use local rainfall data, wherever possible, in analysing yield data for individual areas.

* In analysing the rainfall records, we ignored changes in the position of two gauges. The Hasiheissa gauge was moved 11 miles from Messellemiya in February, 1913. Records were taken at Old Sennar from 1906 to 1928 and at Makwar (Sennar) since 1915; for the 14 years in common we used the means of the two gauges.

TABLE I—MEAN GEZIRA RAINFALLS, 1906 33 INCLUSIVE, IN MM PER ANNUM

(a) Individual Stations. Actual means and means calculated from position

	Lat N. °	Lat N. ′	Long E. °	Long E. ′	Early rainfall: Actual mean	Early rainfall: Calculated mean	Total rainfall: Actual mean	Total rainfall: Calculated mean	Standard deviation: Early	Standard deviation: Total
1—Wad Medani ..	14	24	33	31	47	51	386	365	32	105
2—Manaqil	14	13	32	58	44	43	362	336	32	116
3—Rufaa	14	48	33	19	34	38	323	304	28	109
4—Hasiheisa......	14	38	33	18	34	41	292	321	27	118
5—Kamlin	15	4	33	11	28	29	241	264	28	81
6—Sennar	13	33	33	38	82	72	456	466	43	87
7—Dueim	13	59	32	19	30	34	302	301	22	99
8—Geteina	14	52	32	23	16	16	196	214	17	97
9—Khartoum	15	40	32	34	11	2	160	145	12	94

(b) Five Central Stations (1 5)

	Early	Middle	Late	Total
General Mean	38	222	61	321
S.D.* of mean of 5 stations	23	67	37	82
S.D. per station	30	89	47	107
S.D. per station (eliminating seasonal mean)......	20	64	32	76

(c) All Stations

	Early	Middle	Late	Total
General mean	36	210	55	302
S.D. of mean of 9 stations	18	57	27	71
S.D. per station	28	85	40	101
S.D. per station (eliminating seasonal mean)	22	67	32	76

(d) Regression on Position (all Stations)

	Early	Middle	Late	Total
mm per degree of latitude	−22	58	26	106
mm per degree of longitude......................	22	50	16	90
As percentage of mean per degree of latitude	−60	28	47	37
As percentage of mean per degree of longitude......	60	24	30	30

* Standard deviation.

Records from 1911 were supplied by the Physical Department, Cairo, for the original cotton area at Taiyiba; some corrections were made for the years 1915, 1916, 1917, and 1931 from the records and confidential monthly reports of Sudan Plantations Syndicate, Ltd. Mr. O. W. Snow provided us with a carefully revised statement of the rainfall at the Gezira Research Farm. No records were taken at Barakat during the war years, and we have, therefore, used Wad Medani rainfalls from 1914 to 1918, and subsequently the mean of the rain gauges at the Barakat Headquarters and the Gezira Research Farm, which are at opposite ends of the Barakat area.

The Yield Data

Table II gives the total area cropped and the mean yield of seed cotton in kantars per feddan in each year.* In the early years the irrigated land was concentrated round two pump stations at Taiyiba and Barakat. In 1921 a large extension occurred further south, and we have examined separately the mean yields of the whole of the cropped area since 1911 and, again, since 1921, so as to include a more representative area. The main expansions took place between 1925 and 1930, and most of the land now irrigated has carried only two or three cotton crops. The period since the scheme reached 100,000 feddans of cotton is too short for satisfactory analysis, and most of our discussion must therefore be limited to the older areas.

In order to minimize irregularities from changing rotations and expanding areas at these older farms, we have used the records for individual tenancies to build up mean yields for definite areas, and, in addition, have selected periods for which the cultural methods were most nearly uniform. Thus, we restricted the Old Taiyiba area to the 6000 feddans of land brought into cultivation by 1917, and ignored all subsequent additions to this farm. For this area we made two separate analyses, starting in 1911 and 1917 respectively, since by the latter date the crop rotation had been stabilized. Finally, we selected at Taiyiba a compact block of land, 1440 feddans in area, which was first brought into cultivation in 1917 and cropped in three-course rotation until 1931. This gave five crops on each of the thirds of the individual tenancies, the cotton being grown each year in four long regularly-spaced bands, each of 120 feddans. This set of

* Areas are given in feddans (1 f. = 1·038 acres = 0·420 hectare). Cotton yields are expressed as kantars (315 rotls) of *seed cotton* per feddan (1 k/f = 300·6 lbs per acre = 337 kg per hectare) and, using the conventional factor for ginning-outturn, may be equated to kantars (100 rotls) of *lint cotton* per feddan (1 k/f = 95·4 lbs per acre = 107 kg per hectare).

TABLE II—COTTON YIELDS IN KANTARS PER FEDDAN AND RAINFALL IN MM IN THE EASTERN GEZIRA

Year of sowing	Gezira area in thousand feddans	Total Gezira mean yield	Old Taiyiba	Old Taiyiba 480 fed.	Old Barakat	G.R.F. total	G.R.F. miscel-laneous	Mean rainfall in dm at 5 Central stations			
								Early	Middle	Late	Total
1911	0·25*	5·32	5·32	—	—	—	—	0·65	1·42	0·34	2·42
1912	0·6	5·62	5·62	—	—	—	—	0·24	1·67	0·41	2·32
1913	0·7	3·80	3·80	—	—	—	—	0·05	1·67	0·44	2·17
1914	3·0†	5·29	5·10	—	5·39	—	—	0·42	2·61	0·39	3·42
1915	3·4	3·32	3·10	—	3·48	—	—	0·48	2·26	0·44	3·17
1916	4·3	3·31	3·47	—	3·20	—	—	0·67	2·14	0·68	3·49
1917	3·9	3·29	4·10	5·22	2·66	—	—	0·92	1·69	0·73	3·35
1918	4·0	3·33	4·13	5·38	3·50	—	—	0·59	1·96	0·04	2·58
1919	3·8	5·26	5·60	6·66	4·98	3·25	3·17	0·13	1·76	0·48	2·36
1920	3·7	3·27	3·20	3·59	3·51	2·59	2·34	0·28	3·13	0·68	4·09
1921	9·8‡	3·92	3·52	3·96	3·91	3·05	2·60	0·17	2·42	0·44	3·02
1922	10·4	3·66	4·43	5·20	4·08	3·35	3·54	0·23	3·43	0·38	4·04
1923	22·5§	2·86	2·55	2·56	2·99	3·15	2·86	0·50	3·45	0·45	4·40
1924	21·6	2·21	2·34	2·52	2·29	1·81	1·48	0·23	2·00	0·77	3·00
1925	80·0	4·79	4·32	5·09	3·86	2·45	2·30	0·38	1·52	0·29	2·20
1926	100·1	4·77	4·07	4·20	3·37	3·86	4·08	0·16	1·14	0·53	1·84
1927	105·6	3·29	2·54	2·62	2·89	2·99	2·74	0·17	2·29	1·21	3·67
1928	131·4	3·55	3·74	3·57	3·96	3·13	2·94	0·55	1·53	0·21	2·30
1929	174·1	2·33	1·42	1·85	1·83	2·03	2·21	0·67	3·70	0·63	5·00
1930	196·0	1·34	1·27	1·23	1·51	1·83	1·27	0·43	2·88	0·30	3·61
1931	194·0	4·15	3·76	4·13	3·94	3·12	3·46	0·10	1·95	0·52	2·53
1932	195·0	1·92	—	—	—	—	—	0·53	2·86	0·63	4·02
1933	165·0	2·30	—	—	—	—	—	0·77	2·19	0·80	3·76

* Taiyiba started.
† Barakat. 2000 feddans of cotton. started.
‡ Hosh. 6500 feddans of cotton. started.
§ Wad el Nau. 10.000 feddans of cotton. started.
Land watered by gravitation water from Sennar Dam.

data for 480 feddans annually provides the only long run of uniform cropping of a constant area in the Gezira, and in it the effects of soil irregularity and changing agricultural practice should be reduced to the minimum.

The data for the second pump station at Barakat were limited to the original area of about 6000 feddans. The distribution of cotton within this area was almost uniform, except for two periods (1914–15 and 1919–20) when the cotton was confined to relatively compact blocks. To obtain more uniform material, we have used, in addition to the full data from 1914 to 1931, a restricted period since 1921, during which the cropping was substantially uniform.

At the Gezira Research Farm the exigencies of experimental work on different rotations prevent a simple division of the farm into thirds, cropped in regular rotation, and here, too, expansion brought in new land in the middle of the period considered. There is no single record area with a uniform record of uninterrupted three-course cropping over the whole period. As the best substitutes, we have taken the mean yield for the whole farm, the mean yield for the so-called " water-duty " plots (the continuity of which was broken by bringing in new land in 1925) and the mean yield for a number of miscellaneous plots irregularly distributed throughout the farm for experiments on varieties, sowing dates, and cultural treatments (omitting manuring). In addition, data were examined for two blocks of land which were cropped in alternate years with cotton and either dura, lubia, or fallow. Finally, there were data for one plot of continuous cotton. The Gezira Research Farm data are all for the period 1919 to 1931 inclusive, the first season (1918) being omitted in order that all rotation cotton might have received its appropriate preceding crop.

Table III gives the mean values of rainfall for each of the centres and periods considered, together with the standard deviations for rainfalls in a single season. For convenience of tabulation the rainfalls in Tables II and III and the regression coefficients on rainfall in Tables IV and V are expressed in units of decimetres.

The Effects of Rainfall on Yield

Some indication of the relative importance of rainfall at the four main periods considered in the present analysis is provided by calculating the regression of yield on each of the four rainfall variates: early rain, middle rain, late rain, previous year's rain. These values are given in Table IV for each of the areas selected, together with the mean yield, the standard deviation of one season's yield and the mean annual change in yield. The

TABLE III—MEAN VALUES OF RAINFALL IN DM FOR SELECTED AREAS

Centre	Period	Early	Middle	Late	Previous year	Standard deviations of rainfalls for a single season			
		e	m	l	p	e	m	l	p
1—Total Gezira (5 gauges)	1911–33	0·41	2·25	0·51	3·15	0·24	0·71	0·24	0·83
2—Total Gezira (5 gauges)	1921–33	0·38	2·41	0·55	3·36	0·22	0·81	0·27	0·96
3—Old Taiyiba[1]	1911–31	0·56	2·67	0·60	3·82	0·48	0·98	0·44	1·20
4—Old Taiyiba	1917–31	0·61	2·88	0·67	4·14	0·47	0·97	0·48	1·15
5—Old Barakat[2]	1914–31	0·57	2·75	0·71	4·05	0·33	0·76	0·36	1·01
6—Old Barakat	1921–31	0·49	2·78	0·74	4·18	0·36	0·86	0·32	1·20
7—Gezira Research Farm[3]..........	1919–31	0·40	2·87	0·75	3·98	0·28	1·19	0·26	1·36

NOTES TO TABLES III, IV, V

[1]—*The Old Taiyiba area*

This commenced in 1911 with 250 feddans of cotton on Canals I and II and expanded fairly regularly by the incorporation of new land to about 1700 feddans of cotton in 1917 (" Numbers " 1–39 on Canals I and II and " Numbers " 1–7 on Canals III and IV). The rotations were irregular with considerable areas of wheat, dura, and green crops from 1911 to 1917. From 1917 the rotation was cotton—lubia—fallow until 1925, when half of the lubia was replaced by dura. The area of 1440 feddans, which was cultivated uniformly from 1917 to 1931, consisted of " Numbers " 28–39 on Canals I and II.

[2]—*Old Barakat*

The cotton was grown in a few compact blocks in 1914, 1915, 1919, and 1920, but in other years from 1914 to 1931 cotton was distributed fairly evenly over the area of 6000 feddans. The rotation was cotton—fallow—fallow from 1914 to 1925. Lubia was introduced in 1926, and from 1927 to 1931 the rotation was cotton—half dura, half lubia—fallow.

[3]—*Gezira Research Farm*

A considerable fraction of the three-course cotton at the Gezira Research Farm up to 1931 was in the series of " water-duty " plots :—

Plots	16–22	—	1921	1924	1927	1930
,,	31–37	1919	1922	1926	1929	—
,,	24–30	1920	1923	—	—	—
,,	62–66	—	—	1925	1928	1931

The sequence of cropping was broken in 1925, when the yield on the new plots was quite abnormally low. The previous cotton crop on this land in 1922 yielded more than the average of the Barakat farm of which it then formed part. In 1925, it gave less than one-half of the mean Barakat yield, and even less than the exhausted continuous cotton plots at the Gezira Research Farm. In 1931 the " water-duty " plots were discontinued and, for this season, we took the yields from other experiments on the same plots. The yield of continuous cotton for 1931 was estimated, since the plants were removed deliberately as a precaution against the spread of Blackarm. The approximate areas of cotton per annum were: total farm, 80 to 140 feddans; " water-duty " plots, 25 to 35 feddans; miscellaneous plots, 10 to 50 feddans; cotton in two-course rotation, 2·5 feddans; and continuous cotton, 5 feddans.

TABLE IV—MEAN YIELDS IN KANTARS PER FEDDAN AND REGRESSIONS ON RAINFALL FOR SELECTED AREAS

Area	Period	Mean yield	Standard deviation of season's yield	Mean annual change in yield	Regression coefficients of yield in kantars per feddan on rainfall in dm			
					Early	Middle	Late	Previous year
1—Total Gezira	1911–33	3·60	1·17	−0·102***	−1·66	−0·89***	−1·44	−0·64*
2—Total Gezira	1921–33	3·16	1·10	−0·116	−2·79*	−0·77*	−0·94	−0·43
3—Old Taiyiba[1]	1911–31	3·69	1·20	−0·121***	−1·26*	−0·57*	−1·08	−0·61***
4—Old Taiyiba	1917–31	3·40	1·18	−0·144***	−1·10	−0·49	−0·62	−0·60*
4a—Old Taiyiba (480 feddans annually)	1917–31	3·85	1·51	−0·205**	−1·06	−0·63	−0·92	−0·82**
5—Old Barakat[2]	1914–31	3·41	0·98	−0·081*	−1·68***	−0·26	−1·03	−0·26
6—Old Barakat	1921–31	3·15	0·92	−0·102	−2·00***	−0·39	−0·69	−0 18
7—Gezira Research Farm[3]	1919–31							
Three-course rotation.								
7a—Total farm		2·82	0·63	−0·042	−1·82***	−0·70	−0·51	−0·18
7b—Water duty plots		3·30	1·00	−0·020	−3·09***	−0·16	+0·31	−0·21
7c—Miscellaneous plots		2·69	0·79	−0·025	−2·21***	−0·01	−0·27	−0·31
Two-course rotation. (Old experiments.)								
7d—Cotton-lubia		2·85	0·53	−0·008	−0·75	−0·05	−0·81	−0·00
7e—Cotton-fallow		2·31	0·67	−0·066	−1·55**	+0·02	−0·06	−0·22
7f—Cotton-dura		2·21	0·60	−0·090*	−1·24**	+0·10	+0·34	−0·18
Continuous cotton.								
7g		1·60	0·37	−0·051	−0·77**	−0·10	−0·40	−0·13

TABLE V—PARTIAL REGRESSION COEFFICIENTS OF YIELD IN KANTARS PER FEDDAN ON FOUR RAINFALL VARIATES IN DM AND TIME

Area	Period	Early rain	Middle rain	Late rain	Previous year's rain	Year	Standard errors of partial regression coefficients of yield on				
		e	*m*	*l*	*p*	*t*	*e*	*m*	*l*	*p*	*t*
1—Total Gezira	1911–33	−0·97	−0·62**	−1·22	−0·53*	−0·056*	0·67	0·23	0·71	0·21	0·026
2—Total Gezira	1921–33	−1·06	−0·66*	−1·51	−0·57*	−0·088***	1·09	0·25	0·76	0·20	0·185
3—Old Taiyiba[1]	1911–31	−1·16***	−0·42*	−0·87*	−0·35*	−0·010	0·36	0·18	0·41	0·17	0·037
4—Old Taiyiba	1917–31	−1·32**	−0·37	−0·89	−0·49*	−0·032	0·42	0·20	0·44	0·19	0·052
4*a*—Old Taiyiba .... (480 feddans annually)	1917–31	−1·29**	−0·43	−1·09	−0·64**	−0·078	0·51	0·24	0·53	0·23	0·062
5—Old Barakat[2]	1914–31	−2·20***	+0·07	−1·10**	+0·01	−0·093***	0·45	0·19	0·41	0·15	0·026
6—Old Barakat	1921–31	−2·17*	−0·06	−0·81	−0·09	−0·038	0·73	0·34	1·02	0·21	0·076
7—Gezira Research Farm[3]	1919–31										
Three-course cotton.											
7*a*—Total Farm		−2·07***	+0·16	−1·32***	−0·08	−0·006	0·34	0·07	0·33	0·07	0·021
7*b*—Water duty plots		−3·62***	−0·08	−0·68	+0·07	−0·040	0·72	0·15	0·70	0·14	0·045
7*c*—Miscellaneous plots		−2·43***	+0·17	−1·50***	−0·19	−0·037	0·48	0·10	0·47	0·10	0·030
Two-course cotton. (Old experiments.)											
7*d*—Cotton-lubia....		−1·22	+0·07	−1·18	−0·03	−0·020	0·65	0·13	0·63	0·13	0·040
7*e*—Cotton-fallow ..		−1·50	+0·10	−0·72	−0·11	−0·029	0·72	0·15	0·70	0·14	0·045
7*f*—Cotton-dura		−1·07	+0·13	−0·06	−0·05	−0·065	0·61	0·13	0·60	0·12	0·038
Continuous Cotton.											
7*g*		−0·47	−0·08	−0·65*	−0·12*	−0·037*	0·26	0·05	0·25	0·05	0·016

latter values must be multiplied by three to give the mean change from one cotton crop to the next one on the same land.

It will be seen from Table IV that yields of Barakat and the Gezira Research Farm gave highly significant negative regressions on early rainfall, and that the Gezira as a whole and Taiyiba showed negative regressions on the middle rains, and also on the previous year's rainfall. Since middle rains form the bulk of the year's rainfall, the whole Gezira and Taiyiba are adversely affected by the total rain in both of the seasons preceding the main growth of the crop. These areas also show significant deterioration with time.

The simple regression coefficients are not, however, sufficiently precise measures of the relationship between yield and rainfall, for there may be hidden correlations between the weather at different periods, or progressive changes in rainfall or yield with time. In addition, it is desirable to remove the variance ascribed to one factor before proceeding to test the significance of another.

The best simple expression of the independent effects of the five seasonal variates employed is provided by the partial regression coefficients, that is, by the values for the coefficients k_e, k_m, etc., in the equation

$$(y - \bar{y}) = k_e (e - \bar{e}) + k_m (m - \bar{m}) + k_l (l - \bar{l}) + k_p (p - \bar{p}) + k_t (t - \bar{t})$$

The methods of fitting such multiple regression equations and of testing the significance of the individual coefficients were given by Fisher (1926).

Table V gives the partial regression coefficients (with their standard errors) for yield on the five seasonal variates for the centres already listed in Table IV. These partial regression coefficients form the basis of the discussion in the following sections.

Fig. 2 provides comparisons between the actual yields and those calculated from the multiple regression equations for the total cotton area of the Gezira 1911–33, Old Taiyiba 1911–31, Old Barakat 1914–31, and the total cotton area of the Gezira Research Farm 1919–31.

The size and significance of many of the partial regression coefficients and the general agreement between actual and calculated yields show that a considerable part of the seasonal fluctuation in yield may be ascribed to the effects of varying rainfall. There is clear evidence that a given amount of rain may be more harmful at some seasons than at others, and also that the different centres may respond differently to rainfall. The progressive deterioration is reduced at all but one of the centres by previously eliminating the effects of rainfall. Indeed, at the oldest centre the

deterioration ceases to be significant when the effects of rainfall are removed

All centres show a markedly depressing effect of early rain, and it is interesting to notice that the correlation between yields and early rainfall appears to have been even closer since 1925 than in the earlier years from

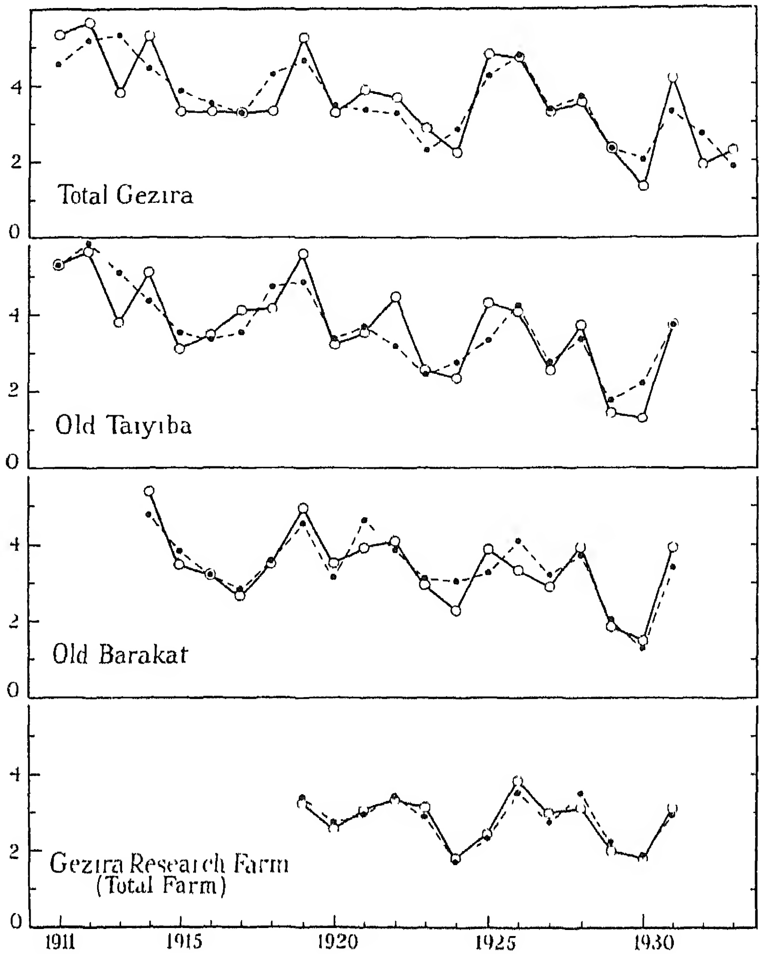


FIG. 2—Comparison of actual yields—o——o—in k/f with yields calculated from multiple regression equations on rainfalls and time—•——•

which it was first detected Most centres show depression from late rains, but they differ in their responses to middle and previous year's rain.

In comparing different centres and periods, it should be noted that as they include certain common years, they are not independent. Further, the standard errors of the coefficients are relatively high for the shorter periods

The partial regression coefficients on rainfall are expressed in kantars of cotton per feddan per decimetre of extra rainfall, but in considering the scale of these rainfall effects, it must be remembered that in absolute amounts both early and late rains are much smaller and less variable than the middle or total rains. The general magnitude of the effects due to rainfall may be illustrated by calculating the depressions in yields due to certain postulated rainfalls, taking as standards the partial regression coefficients for the 480 feddans annually at Taiyiba from 1917 to 1931.

TABLE VI—REDUCTION IN YIELD, KANTARS PER FEDDAN, BY EXTRA RAINFALL, CALCULATED FROM COEFFICIENTS FOR TAIYIBA, 480 FEDDANS, 1917–31

Amount of extra rainfall	Early	Middle	Late	Previous
1—100 mm . .	1·3	0·4	1·1	0·6
2—Taiyiba means 1917–31	0·8	1·2	0·7	2·6
3—Standard deviation of mean Central Gezira rainfall	0·4	0·4	0·5	0·6

From the values in Table VI, it will be seen that, although unit early rainfall has three times the effect of unit middle rainfall, doubling the mean rainfall at each of the four periods would have much more effect for middle or previous year's rain than for the two other periods. Such wide changes in rainfall are, however, much less common for middle and total rainfall than for early and late rainfall, and the best general illustration of the relative importance of the four periods is obtained by considering amounts of extra rain equal to the standard deviation of rainfall per season for the five Central Gezira stations (viz., early 30 mm, middle 90 mm, late 50 mm, total 100 mm). These amounts of extra rainfall lead to the values in line 3 of Table VI, which are of the same order for the four periods.

It happens that the mean rainfalls for Taiyiba between 1917 and 1931 are similar to the means of Wad Medani and Sennar for the period 1906 to 1933, which may be taken to represent the mean rainfall of the extreme south of the present irrigation area. The extreme north (*e g*, Kamlin) has about half as much rain as the south, and should, therefore, possess an advantage due to its position of about one-half of the amounts tabulated in line 2 of Table VI. Lower rainfall probably accounts in part for the continued success of the northern area in spite of its admittedly poorer soil. Projected schemes in the north-west around Dueim and Geteina would also appear to be favoured by the low rainfalls at these places.

The above illustrations were based on results for a single centre and period, but Table V shows that the partial regression coefficients differ in magnitude and significance between centres The effects of rainfall at the separate periods are therefore considered in more detail in the following sections.

Early Rains

In all of the data examined for three-course cotton at single centres, the early rains reduce the yields significantly, at the rate of from one to over two kantars of seed cotton per feddan per decimetre of rain. Although some of the low yields might be ascribed in certain years to particularly severe Blackarm or Leaf Curl damage, and in others to excessive flooding or other causes, it should be emphasized that the early rainfall is the only factor which has been successfully related to the yields at the three older centres over a long period of years The early rainfall effect fails to reach significance for the Gezira as a whole, probably on account of the lower variance for the means of five widely separated rain gauges, and the irregularities in soil and rainfall over a large and increasing area

The effects of the first rains on the physical, chemical, and biological properties of the soil were not investigated in detail until recently, for it was generally assumed that May and June were unimportant months for a crop which was to be sown in August after much heavier rain and irrigation. Partly as the results of the present analysis, they are now receiving active study, and we do not propose, therefore, to discuss the possible mechanism of the early rain effect in any detail. It will be sufficient to refer to some of the hypotheses advanced The essential feature of the first appreciable rainfall is that it interrupts the desiccation of the soil which proceeds through the fallow period. Early rainfall shortens the fallow, and any explanation of the effects of early rains requires an understanding of the effects of fallow on the Gezira soil

In 1925, E. M. Crowther (1926) suggested that the early rains initiated the cycle of biochemical oxidation in the soil, and postulated that micro-organic activity long in advance of the main rains and first irrigation led to an increased loss of available nitrogen from the wet soil during July and August. It has, however, recently been shown (F Crowther, 1933–34) that flooding soil rich in nitrate does not necessarily cause a permanent loss of available nitrogen On removing the surplus water, the nitrate content soon rises rapidly, either through nitrification or through capillary rise in the wet soil Flooding before the cotton was planted had no adverse effect on the growth and final yield of cotton

F. Crowther (1933–34) suggested that early rains affected the crop through causing some adverse condition in the subsoil. It was repeatedly observed that, in years of early rainfall, the crop grew well until November and then fell back rapidly, as would be expected if root growth in the second and third feet of soil was restricted by some shortage of water, oxygen, or nitrate. The Gezira soil cracks extensively during the fallow seasons, and F. Crowther suggested that the early rains run down and close up these cracks from the bottom upwards, thus making the accessible soil profile still shallower. In a preliminary experiment in 1933–34 on screening small plots from early rains, he obtained significantly higher mean yields of cotton per plant than on unprotected soil. Snow (1933) suggested that the lower temperatures and higher humidities in May and June in years of high early rainfall reduce evaporation and thus prevent further desiccation and cracking. It is well known that the yields fall off rapidly as the sowing date is postponed after an optimum about the middle of August, and Greene (1933–34) suggested that the optimal sowing date may be related to the time of the first rain which initiates the main seasonal cycles of physical and biological changes in the soil. Effects of early rains in enabling weeds and insects to survive the later and more intense stages of the fallow and in favouring " wilt " and other soil fungi have also been considered. But none of the above hypotheses has yet been substantiated.

Mid-Season Rains

About three-quarters of the total annual rainfall occurs in July and August, and during this period the land is irrigated and the seed sown. Heavy rains delay sowing and may necessitate resowing. They wash out quantities of seed and delay work on the land; young cotton plants may be asphyxiated by flooding, and weed growth is encouraged at a critical period. Late re-sowings give low final yields, for it has been repeatedly demonstrated that September sowings give much lower yields than August sowings.

The analysis in Table V shows that high mid-season rainfall reduces the yield in the Gezira as a whole, and at Taiyiba, but has no appreciable effect at Barakat or the Research Farm. The differences in standards of cultivation and supervision would lead one to expect less disturbance from flooding at the Research Farm than over the large commercial area, which, in addition, contains many low-lying parts from which it is difficult to draw off surplus water. The Taiyiba and Barakat farms are similarly managed, and the difference between them probably depends on the level

of the belt of land which separates them from the Blue Nile. At the Barakat farm where mid-season rains are without effect, this land is relatively low and flood water is more easily drained away than at Taiyiba, where the intervening land is higher and the depressing effect of mid-season rains is significant

Late Rains

Rainfall in September and October is negatively correlated with yields for the full term of years at Taiyiba, Barakat, and the average of the Gezira Research Farm The regression coefficients are of similar magnitude, though not significant, for the whole Gezira, for shorter periods at Taiyiba and Barakat, and for the water duty, cotton-fallow, and cotton-lubia plots at the Gezira Research Farm.

It is known from Massey's (1930, 1931) work on Blackarm that rain splashes spread the bacteria responsible for the secondary infection, and that the disease can develop rapidly in the plant tissues only under the humid conditions associated with actual rainfall Late rains greatly increase the damage to affected plants Late rains may also cause temporary flooding when they coincide with irrigation. Watering proceeds continuously throughout the Gezira from October onwards at about fortnightly intervals at any one point The small amounts of rain in October may increase the risk of flooding by puddling the soil and by reducing evaporation through the increased humidity and cloudiness associated with rain. Flooding and over-watering during the early stages of growth cause the leaves to turn yellow, and F Crowther (1933–34) showed by direct experiments in 1932 and 1933 that such flooding greatly reduced the final yield, probably through root asphyxiation.

The fact that at the Gezira Research Farm, the total farm, the miscellaneous plots, and continuous cotton show large late-rain effects suggests that the increased development of Blackarm due to these late rains may be more important than the physiological effects due to waterlogging. The miscellaneous plots always include variety and sowing-date experiments, both of which are notoriously liable to the early appearance of Blackarm which invariably causes severe damage on these plots The variety experiments often include infected Gezira seed, and early sowing renders cotton particularly susceptible to damage. Since 1925, the miscellaneous plots have taken the form of replicated and randomly-distributed small plots These favour the spread of Blackarm, which is essentially an "edge-effect" On the other hand, in the "water duty" and the two-course rotation plots, which have insignificant late-rain effects, the cotton occurs as single blocks of similarly treated plants Great

emphasis should not, however, be placed on these differences in response to late rainfall, for the differences between the regression coefficients are subject to relatively large errors It is probable, too, that the bad effects of late showers will depend more on the intensity of the storms than on the total amount of rain in two or three months

Previous Year's Rainfall

The rainfall in the previous season (10 to 16 months before the cotton is sown) is negatively correlated with yields in the Gezira as a whole, at Taiyiba and in the continuous cotton at the Gezira Research Farm, but not at Baraket or the rest of the Research Farm.

This grouping of centres suggests that the previous year's rains influence the cotton crop chiefly by increasing weeds, and that the effect is more serious on land which has been frequently cropped and irrigated. At first, the Taiyiba farm was cropped every year, and throughout the rest of the period considered each cotton crop was followed by irrigated dura or lubia At Barakat, successive cotton crops were generally separated by complete fallows for two years Frequent cropping and irrigation would favour weed growth, and they might also cause some deterioration in soil structure Heavy rainfall in the year preceding a cotton crop would leave more weeds and weed-seeds, and, in addition, more dried straw-like residues which would immobilize available soil nitrogen when the young cotton most needed it In 1931 and 1932, food crops were omitted from the whole of the commercial cotton area, and from 1933 the rotation was changed to four-course to allow a two years' fallow before each cotton crop and in the hope of providing greater control of perennial weeds.

Deterioration of Soil and Weather

The low yields of the last few seasons and the general downward trend of the mean Gezira yield, since the dam began to be used, have sometimes been taken by others (Balls, 1935, Vageler and Alten, 1932) as evidence of serious soil deterioration It is generally believed that virgin land yields more than previously irrigated land, but it is a matter of the utmost importance to know whether there is evidence of further progressive deterioration In the past, many irrigation schemes have failed through the deleterious effect on the chemical and physical properties of the soil of irrigation without drainage Again, the cultivation of a single new crop in a large and isolated area might allow a rapid spread of

certain weeds and pests, especially when seed is imported. Current research and observations on these possibilities need to be supplemented by an analysis of slow changes in the recorded yields of definite areas, after eliminating the estimated effects of other known variables. The simple regression coefficients of yield on time, ignoring rainfall effects, Table IV, are significantly negative for the whole Gezira area, for Taiyiba, and probably for Barakat. They amount to about one-third of a kantar per feddan, and about 10%, for each cropping rotation. It must, however, be remembered that in the early years the Taiyiba farm formed either the whole or a large fraction of the total cotton area, and that in spite of the significant decline, its mean yield is still high. Barakat and the Research Farm gave lower mean yields and deteriorations, as would be expected from the circumstance that they are both near the large town of Wad Medani and were overcropped with dura before the irrigation scheme. Expansion from land of the Taiyiba type to the Barakat type would produce apparent deterioration of the overall yield, and it is known that the present irrigated area contains some land of still lower inherent fertility.

A run of seasons with unfavourable weather or a progressive increase in rainfall could account for falling yields in short sets of data. The partial regression coefficients of yield on time in Table V show the progressive changes in yield, calculated for constant values of the four rainfall variates considered. Deterioration was significant only for the total Gezira area, the full term of Old Barakat and the continuous cotton plots of the Gezira Research Farm. The behaviour of the oldest centre, Taiyiba, is of the greatest importance. Correction for rainfall effects reduces the mean annual change over the whole area and period of this farm from $-0{\cdot}12 \pm 0{\cdot}04$ k/f to $-0{\cdot}01 \pm 0{\cdot}04$ k/f, and for the five rotations of uniformly treated cotton on 480 feddans annually from $-0{\cdot}21 \pm 0{\cdot}07$ k/f to $-0{\cdot}08 \pm 0{\cdot}06$ k/f. Thus, the deterioration ceases to be significant after allowing for rainfall effects. The pronounced downward trend in the Taiyiba yields apparent from fig. 2 is to be ascribed to the combination of marked effects of rain in the two seasons preceding the cotton crop with the fact that the Taiyiba rainfalls increased significantly over the period considered. There is no direct evidence that the chemical and physical properties of the soils have been adversely affected by irrigation at the older centres, but before ascribing the deterioration to the weather rather than the soil, it seems necessary to inquire whether other rainfall data support, for once, the practical man's opinion that the weather is not so good as it was.

Progressive Changes in Rainfall

During the period 1911 to 1931 the total rainfall at Taiyiba increased significantly at the rate of $10{\cdot}4 \pm 3{\cdot}6$ mm per annum. This was made up of increases in early, middle, and late rainfalls of 2·2, 5·9, and 2·5 mm per annum respectively, which were not, however, significant when considered individually. The nearest rain-gauge (Wad Medani) showed no such increase in total rainfall during the same period, and one might be inclined therefore to regard the apparent change at Taiyiba as accidental or as due to some increasing disturbance, such as a growing tree or changing buildings. Although it happens that the Taiyiba gauge was moved two miles about 1917, inspection of the past and present sites of the gauge revealed no cause for a progressive disturbance, and no reason to doubt the readings. It happens that the Taiyiba and Wad Medani gauges provide the extreme values for the progressive change in rainfall. The other eight stations all gave increasing readings during 1911–31. At Sennar the annual increase amounted to $6{\cdot}0 \pm 2{\cdot}2$ mm for total rainfall and to $4{\cdot}0 \pm 1{\cdot}3$ mm for early rainfall, both of which were highly significant. (The progressive increase cannot be ascribed to the change from Old Sennar to Makwar, for Old Sennar had the higher mean rainfall during the 14 years in common.) In middle rains all nine stations showed increases—mean value 3·0 mm—and at Rufaa the increase of $6{\cdot}2 \pm 2{\cdot}9$ mm was significant. With such additional evidence of increasing rainfall at other centres in the Gezira, there would appear to be no reason for doubting the validity of the increase in rainfall at Taiyiba. There were no other significant changes in early, middle, late, or total rainfall during 1911–31, but at Wad Medani there was an almost significant decrease in early rainfall of $2{\cdot}1 \pm 1{\cdot}1$ mm per annum.

Periodic Changes in Rainfall

Oddly enough, it happened that both the beginning of the scheme at Taiyiba in 1911 and the opening of the dam in 1925 occurred during spells of relatively low rainfall. For a few years before 1911, the rainfalls were so high that over the whole period for which data are available (1906–33) there is no evidence of any significant change in total rainfall at any of the nine rainfall stations. The only significant increase with time found in testing the three periods of rainfall and their totals at each of the nine stations was for early rainfall at Sennar, $+2{\cdot}5 \pm 0{\cdot}9$ mm per annum. The progressive increase over the shorter period, 1911–31, is presumably the result of some obscure periodicity, and there is no reason to ascribe it

to the effects of the dam and irrigation, or to expect that it will continue

A run of 28 years is insufficient for any elaborate analysis of periodicities, but it is of interest to notice that the Gezira rainfall records from 1906 to 1933 show a significant seven-year periodicity with minima about 1912, 1919, 1926, and 1933 Table VII gives the coefficients of simple harmonic motion for this periodicity at the nine rainfall stations, together with the amplitude or maximum fluctuation on either side of the mean value

TABLE VII—SEVEN YEARS' PERIODICITY IN GEZIRA TOTAL RAINFALL

Station	Co-ordinates of		Amplitude
	$\cos \frac{2\pi t}{7}$	$\sin \frac{2\pi t}{7}$	
1—Wad Medani	−62	28	68*
2—Manaqil	−74	−18	76*
3—Rufaa	−47	−11	48
4—Hasiheisa	−79	−21	82*
5—Kamlin	−57	24	62**
Mean of 1–5	−63	0	63***
6—Sennar	−16	−11	19
7—Dueim	−1	−15	15
8—Geteina	−57	+17	60
9—Khartoum	−38	+36	52
Mean of 1–9	−48	+3	48*

For the mean of the five central stations the significance of the seven-year periodicity in total rainfall reaches the 1% probability value, and at four of these stations considered individually the significance reaches the 5% point The fitting is not significant for the outlying stations taken individually, though it remains significant for the mean of all nine stations. Fig. 3 illustrates the periodicity in the total rainfall for the means of the five central stations by a comparison of the curve fitted to the annual values and lines joining three-year means

Although a seven-year term does not correspond to any of the well-known meteorological periodicities, there is additional evidence to support it in the Sudan. Thus, the Secretary for Economic Development, Sudan Government (1931–32), wrote in 1932 " From the records that are available, the periods of food scarcity appear to occur in cycles of six or seven years In 1913–14, 1918–19, and again in 1925–26 measures had to be taken by the Government to meet a serious shortage in food grains. Between these lean years occur the seasons of plenteous supply, when the

country has a surplus of grain for exports " Similar relief measures were required in 1933 Each of these four periods is within a year of the minimum in the seven-year rainfall cycle illustrated in fig 3 The desperate famine of 1889 and the only other one recorded (1903) are both within two years of minima on the same cycle The bottom curve of fig 3 shows that the dura exports from the Sudan exhibit some seven-year periodicity, though the amplitude naturally depends on external economic factors The cotton yields in the Gezira tend to be high during the spells

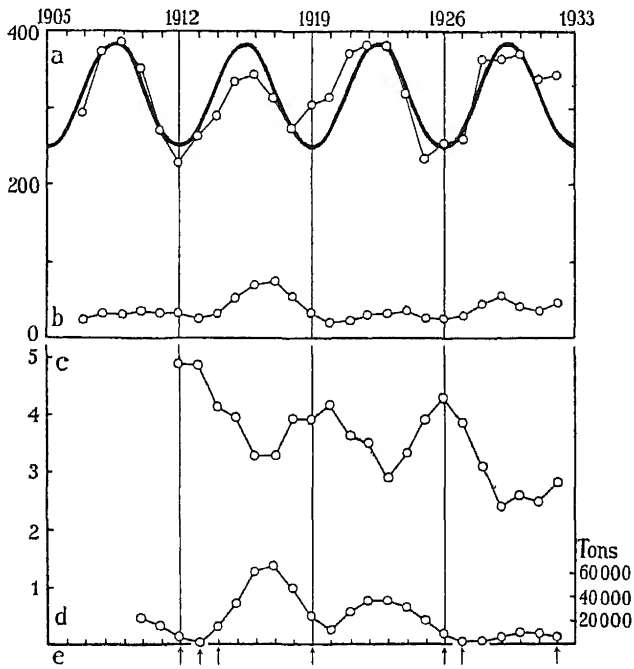


FIG 3—Three-year means for *a*, total, and *b*, early rainfall in mm for the average of five East-Central stations, *c*, average cotton yield in k/f for the whole Gezira area, *d*, total exports of dura from the Sudan The thick line in *a* gives the calculated seven-year periodicity for total rainfall The arrows, *e*, mark years in which dura exports were below 2,000 tons

of low total rainfall On the other hand, there is some suggestion that the total cotton production at Tokar is lower during these periods, the area planted is controlled by the flood of the River Baraka, which in turn depends on the rainfall in the Red Sea Hills

In the early and total rainfall of the Gezira there is some sign of a fourteen-year periodicity, but the data are insufficient for an adequate test

These periodicities are not sufficiently well established to be of much

value in forecasting future rainfalls, but they are sufficient to show the value of a systematic collection of reliable rainfall records at as many places as possible, especially during the early stages of new agricultural developments When taken in conjunction with the rainfall correlations presented earlier in this paper, they suggest that the declining yields in the early years at Taiyiba, and again two cycles later on the expanded scheme, may have been due in part to a temporary worsening in the weather and not necessarily to soil deterioration or the inadequacy of the rotations and cultural methods then adopted

The Need for Record Areas

We suggest that the detection of possible effects of weather and of soil deterioration is sufficiently important to justify the retention of uniformly treated record areas on an adequate scale in new agricultural schemes Thus, we regret that it was found necessary in 1932 to change the rotation on the 1440 acres of Taiyiba which had been farmed for fifteen years on a uniform plan. Should the correlations observed in the past fail to be repeated during the next decade or two, it will be impossible to decide whether the effects so far observed were accidental or whether they were valid only for a specified system of cultivation, since abandoned. In irrigation schemes it is particularly important to ascertain by direct trial the direction and approximate rate of progressive changes in soil fertility. The commercial advantage of treating the whole of a large area uniformly may be dearly bought if attempts to use the latest suggestions from practice and experiment are allowed to destroy the means of measuring and interpreting the value of those changes and of detecting the more fundamental causes of variations in yield.

We wish to record our indebtedness to the Sudan Plantations Syndicate, Ltd , the Controller of the Gezira Agricultural Research Service, the Director of the Egyptian Irrigation Department, Khartoum, and the Director of the Physical Department, Cairo, for affording us access to their detailed records, and for permission to publish our findings We have profited from many discussions with Messrs M A Bailey, H. Greene, and O W Snow of the Gezira Agricultural Research Service on the interpretation of the results, and with Mr. F Yates, of Rothamsted Experimental Station, on the methods for the statistical analysis; we are also indebted to them for detailed criticisms of a draft of this paper.

Summary

The relationships between seasonal yield and weather fluctuations for cotton grown under irrigation in the Sudan Gezira were analysed for periods up to 23 years

The analysis confirmed the generally recognized bad effects of high rainfall about the period of sowing cotton, but showed that this effect was not universal An unsuspected but apparently general effect was discovered Cotton yields were negatively correlated with the amount of early—May and June—rainfall In some areas yields were negatively correlated with late rainfall and with the total rainfall in the preceding year The differences between areas in their responses to weather could be partially interpreted in terms of their situations and agricultural histories.

The progressive decline in yield at the oldest trial farm could be largely accounted for by a significant increase in total rainfall during the period of cotton cultivation. The total annual rainfall in the Sudan Gezira exhibited a significant seven-year periodicity, which was reflected in cotton yields, dura exports, and recorded famines It happened that the first trial of irrigated cotton and the first use of the Sennar Dam coincided with minimal rainfalls on this periodicity. The early promise and rapidly increasing difficulties may well have been due in part to the recurrence of unfavourable weather conditions and not necessarily to soil deterioration and pests

Uniformly treated record areas could easily be established on a normal commercial basis in irrigation projects under central control, and would provide valuable material for research in agricultural meteorology, and a surer background for determining both experimental and commercial programmes

References

Balls, W L (1935) 'Emp Cotton Grow Rev,' vol **12**, p 32.

Crowther, E M (1926) 'Sudan Govt Rep of a meeting in the Sudan Gezira in December, 1925, for the discussion of certain problems connected with cotton growing,' p. 18

Crowther, F. (1933) 'Sudan Govt Ann Rep Gezira Agric Res Ser,' p 71.

—— (1933–34) 'Sudan Govt. Interim Rep Gezira Agric Res Ser.,' p 25.

Fisher, R A (1924) 'Phil Trans,' B, vol **213**, p 89

—— (1926). "Statistical Methods for Research Workers," Edinburgh, Sec. 29.

Greene, H (1928, *a*). 'J. Agric Sci,' vol **18**, p 518

—— (1928, *b*) 'J. Agric Sci,' vol. **18**, p 531.

Greene (1933–34) 'Sudan Govt Interim Rep Gezira Agric Res Ser,' p 50
Hewison, R (1931) 'Emp Cotton Grow Rev,' vol. 8, p 290 *a*
Massey, R. E (1930) 'Emp Cotton Grow Rev,' vol 7, p 185
—— (1931) 'Emp Cotton Grow Rev,' vol 8, p 187
Snow, O W (1933) 'Sudan Govt Ann Rep Gezira Agric Res Ser,' p 106
Sudan Government (1931–32) 'Ann Rep Sec for Econ Devel.,' vol 25, p 3
Vageler, P, and Alten, F (1932) 'Z Pflanz Dung,' A vol 23, p 1

576 312 32

The Internal Mechanics of the Chromosomes IV—Pairing and Coiling in Salivary Gland Nuclei of *Drosophila*

By P C Koller

(John Innes Horticultural Institution, London)

(*Communicated by Sir Daniel Hall, F R S—Received May* 2, 1935)

[Plates 22–25]

1—Introduction

The most urgent problem in dealing with the salivary gland chromosomes is to find out their relationships with ordinary mitotic and meiotic chromosomes—first in structure and secondly in movement. Others have already dealt with structure in some respects, but movement has been generally neglected.

It has been known since the work of Balbiani in 1881 that the chromosomes in the resting nuclei of salivary gland cells in the larvae of Diptera appear as enormously enlarged threads. These chromosomes are cylindrical threads consisting of transverse bands of deeply-staining material separated by non-staining internodes. During the last two years it has been found possible to relate this structure with the genetic properties of the chromosomes Heitz and Bauer (1933) showed in *Bibio hortulanus* that these cylinders were the product of the pairing side by side of two cylinders corresponding in structure. The pairing is analogous with that observed in the zygotene stage of meiosis in *Tulipa* and *Stenobothrus*, where chromomeres correspond Painter (1933) showed the even more significant fact that the banded structure was constant and characteristic of individual chromosomes of *Drosophila melanogaster* Further Muller and Prokofyeva (1935) have shown by deletions with X-rays in *Drosophila* that the individual band or part of a band corresponds with a gene Finally, it seems probable, from the observations of Koltzoff (1934) and Bridges (1935), that in the paired thread each band represents the reduplication of a chromomere or gene by division. Apparently the inert regions of the chromosomes in the neighbourhood

of the attachment constriction are not differentiated at this stage, but appear, together with the spindle attachment chromomere, as undifferentiated masses which later become fused into a single body This body seems to me to be optically homogeneous in the living condition, and I shall therefore refer to it as the *magma*

The importance of these discoveries for the genetic analysis of the *Drosophila* chromosomes has been generally recognized Elsewhere I have attempted to use them for this purpose (Koller, 1935, *a*) The problem of their mechanical relationship with the chromosomes seen in the normal nuclear cycle has not yet been considered In fact, in the course of the most recent studies every effort has been made to obliterate by pressure the properties of spatial arrangement of these chromosomes, whenever they hindered the straightforward genetical analyses Yet it is clear that the enormous size of the chromosomes in these nuclei gives an opportunity, such as can be found nowhere else, of determining essential properties of the chromosomes

It seems that we can regard these chromosomes as corresponding with paired pachytene chromosomes at meiosis in which the intercalary parts between chromomeres have been stretched and separated into smaller units. and in which, instead of two threads lying side by side, we have 16 or even more Hence they are "polytene" rather than pachytene, I do not, however, propose to use this term, I shall refer to them as "multiple threads" Further, it seems that the nuclei must be regarded as resting or prophase nuclei, and we may therefore look for a structural similarity between the arrangement of the chromosomes found in these nuclei and that found in the ordinary resting or prophase nucleus

We may consider briefly the normal structure, as shown in the first three studies of this series (Darlington, 1935) The chromosomes are lying packed in the nucleus as threads, wound in regular spirals These are the *relics* of the spiral coils in which the threads were wound at the previous metaphase They have been partly uncoiled during telophase, and they continue to uncoil during the prophase When—as in meiosis—corresponding chromosomes pair before the internal or molecular changes which determine this uncoiling are complete, the paired chromosomes proceed to coil round one another to form *relational* spirals My present object is therefore to find out whether the relic and relational coils are represented in the nuclei of the salivary gland cells, and whether they reveal the co-ordinated movements of chromosomes, which are consistent and characteristic during mitosis and meiosis

2—Material and Technique

The salivary glands of larvae of *Drosophila melanogaster*, *D pseudo-obscura*, *D sub-obscura*, *Chironomus* sp , *Calliphora* sp , as well as hybrids of *D pseudo-obscura* races (Koller, 1935, *b*), were used as material The technique generally employed was that described by Painter (1934) with some modification The salivary glands were left from 5 to 10 minutes in the aceto-carmine solution, without a cover slip, exposed to evaporation, which was helped sometimes by warming one side of the slide slightly When the salivary glands were darkly stained, they were covered with a cover-slip and pressed slightly or strongly according to the purpose for which the preparations were required

Smeared preparations were made with medium Flemming solution and stained with gentian violet The structure of the chromosomes was clearer in this case than with aceto-carmine, but the Flemming technique had the great disadvantage that the individual chromosome threads outside the nucleus were distorted and useless for study However, this method was greatly improved by pressing the salivary glands in 45% acetic acid, and leaving them in this for 5–10 minutes, after which the fixation was continued in medium Flemming solution The Feulgen reaction was employed to detect the distribution of chromatin

The behaviour of the salivary gland cells was also studied in the living condition It was possible to keep these cells in frog-Ringer solution for about 3–5 hours By slight pressure, nuclei were separated from the cell, and their contents were made visible with methylene blue vital stain The dilution of stain was approximately 1/1000

The drawings were made with the help of a camera lucida, using a Zeiss 40 × objective and 20 × eyepiece, giving a magnification of 2500, which is reduced in the text

I am very much indebted to Mr L La Cour for his help with the technique and to Mr H C Osterstock for taking the photographs with a camera of his own construction

3—The Structure of the Chromosomes

When the chromosomes are drawn out flat with aceto-carmine technique without employing excessive pressure, they are from 80 to 100 times the length of the mitotic metaphase chromosomes That is to say, the X-chromosome of *D pseudo-obscura*, which in mitosis is 2 8 μ long, in the salivary gland is 240–260 μ in length In this chromosome I counted about 660 staining bands or discs These vary in diameter from 3 to 5 μ, but

the variation seems to be less in living material, the larger discs having probably extended more with aceto-carmine treatment. In thickness the discs vary from 0 5 to 3 μ, the thicker ones are the result of several bands coming together, fig. 1 and fig. 26, Plate 23, as a result of the same swelling process These bands can be separated into their constituents by pressure The total number of units is therefore in the neighbourhood of 1000 in the X-chromosome The total number of simple and compound bands counted in the whole complement was about 2500, which would actually represent about 4000 units The calculation is in agreement with that of Muller and Prokofyeva (1935), assuming that some of the bands contain a cluster of genes rather than individual genes

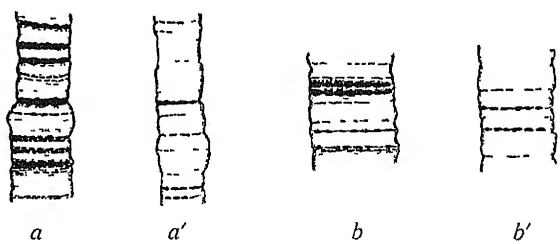


FIG 1—Identical segments of chromosomes in the salivary glands of *D pseudo-obscura*, *a* and *b* slightly, *a′* and *b′* strongly pressed preparations

The internodes between the bands vary in length from 6 μ down to the limit of visibility The finer bands can be seen to be made up of globular particles This structure of the fine bands is clearly visible with medium Flemming fixation, which does not swell the particles, fig 19, Plate 22. In larger, thicker bands, the individual particles cannot be seen, only the crenate boundaries of the band indicate the presence of the globular constituents, fig 20, Plate 22.

It seems, therefore, that the same kind of structure can be assumed for all the bands, the difference in appearance depending on the size of the particles and on whether they are multiple or not, fig 1

It is possible to show in heated aceto-carmine preparations that the particles of the successive bands are connected by fine threads, fig 18, Plate 22. We can see they are effective connections by the fact that, if a portion of one band has been slightly displaced in the course of treatment, the corresponding part of the next band is similarly displaced fig. 2, *a* and *b*. The fibrous structure of the chromosome may be seen at points where the thread is half broken, and remains together in the other half, fig. 2*c* The cases of twisting, fig 2*d*, are further proofs of the longitudinal cohesion of these intercalary fibres

The number of globular particles in the multiple threads was counted in different Diptera, and is given in Table I

The number of granules probably indicates the number of fibres present in the compound threads These fibres may be arranged peripherally as Koltzoff (1934) has supposed, or evenly throughout the cross-section of the cylindrical chromosome

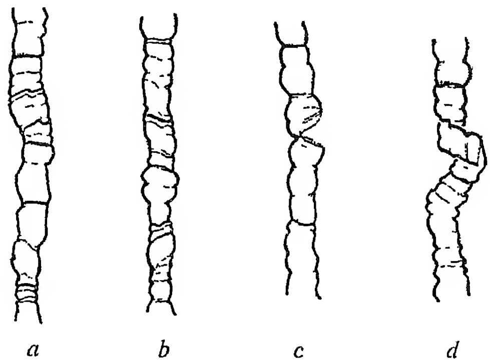


FIG 2—Chromosomes showing (*a* and *b*) displacement of successive bands (*c*) partial breakage, and (*d*) twisting

TABLE I—NUMBER OF GRANULES IN INDIVIDUAL BANDS OF SALIVARY GLAND CHROMOSOMES

Species	Minimum number	Average	Maximum number	No of bands counted
Drosophila melanogaster	8	16	20	35
Drosophila pseudo-obscura	12	16	24	50
Drosophila pseudo-obscura hybrid	8	16	22	50
Chironomus sp .	16	16	30–32	13

The increase in size observed after pairing of the homologous threads, Table II, shows that the fused, multiple chromosomes have the diameter expected if their constituent fibres are distributed throughout their cross-section and not only at the periphery, and one may assume that the cylinder is solid, fig 3.

The discs are composed of granules lying at the same level, either within or on the surface of the cylinder, and pressed together, giving the appearance of continuous or discontinuous bands, according to the size of the individual particles, fig 4

4—THE DEVELOPMENT OF THE CHROMOSOMES

The observations—as enumerated above—suggest that the chromosomes of the salivary glands are compound, each thread consisting of

TABLE II—THE DIAMETER OF UNPAIRED AND FUSED MULTIPLE THREADS OF SALIVARY GLANDS IN *D pseudo-obscura*

	Unpaired observed		Paired					
			Observed		Calculated			
					Minimum		Maximum	
	Minimum	Maximum	Minimum	Maximum	Solid	Hollow	Solid	Hollow
	μ	μ	μ	μ	μ	μ	μ	μ
a	3	5	4	6 5	4 2	7	6	10
b	—	4 5	—	6	—	—	6 3	9

(*a*) Measurement of identical segments in the same chromosome of several nuclei.
(*b*) Measurements taken at the point of fusion

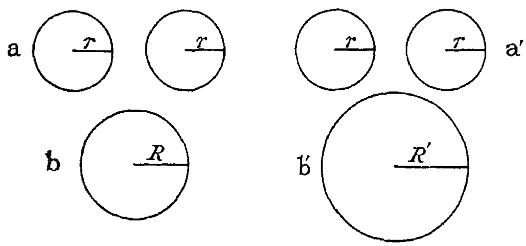


FIG 3—Diagram illustrating the diameter of chromosome threads *a*, *a'* before, and *b*, *b'* after fusion, *b* diameter of fused threads if the cylinder is solid; *b'* diameter if it is hollow. R = 1 4*r*, R' = 2*r*

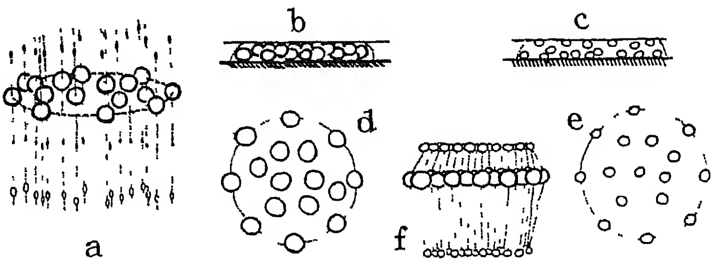


FIG 4—Diagram illustrating the structure of the multiple threads, *a* and *f* the arrangement of fibres with chromomeres of different size in side view, *d* and *e* cross-section of thread at different level with large and small chromomeres, *b* and *c* the result of smearing, giving the appearance of continuous and discontinuous bands

several fine fibres (gene-strings) which are evenly packed inside or on the surface of a cylinder. They are separated in the internodes between bands, but come into close association at the discs The single fibres show similar structure at the same level, which indicates their common origin. The question then arises, how do such compound structures develop ?

A detailed embryological study of the salivary gland is difficult in young larvae of Diptera, but several instances were found in old larvae where cells showed different stages of development In these larvae, the successive processes of general differentiation could be followed

After the last mitotic division the cells of the future salivary glands enter into a long resting period Later, a general growth of the complete cell can be observed, both the cytoplasm and the nucleus increase in volume in the same degree, fig 5*a* and *b* In the living state, the cytoplasm contains numerous globules which move freely Their number rapidly increases These globular bodies are most probably enzymic

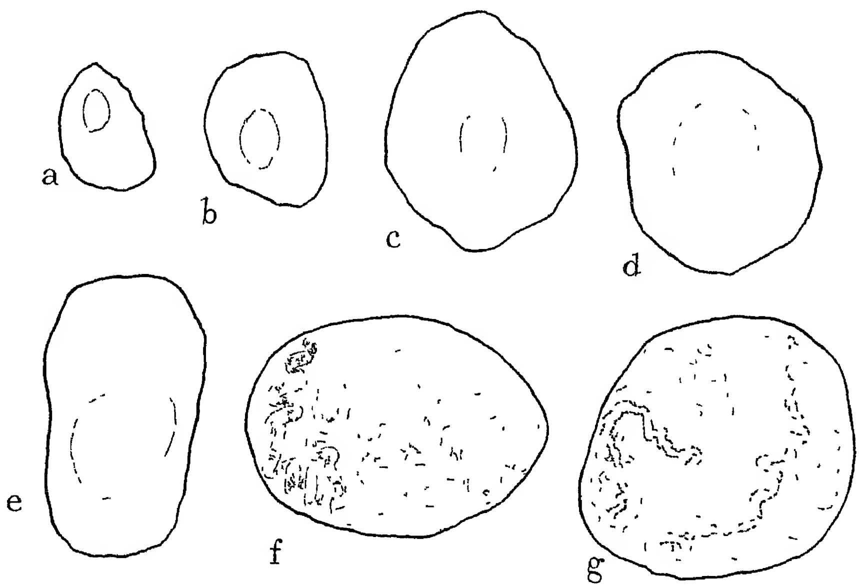


FIG 5—Camera lucida drawings illustrating the successive stages of development of the salivary gland cell

products. In aceto-carmine preparations it is rather difficult to observe the globules, as the cytoplasm and its contents stain evenly. The nucleus contains fine threads, which form a compact knot The threads are thin and slightly stained

At the next stage, fig 5*c*, the cytoplasm increases without an observable growth of the nucleus The number of globules in the cytoplasm is enormous, the cytoplasm is entirely filled up with them Following this, fig 5*d*, there is an increase in the nucleus The chromosomes are more clearly visible, showing twists, and sometimes it is possible to follow individual chromosomes along their length, fig 5*f*, and fig. 36, Plate 24

This stage, the first at which signs of polarization can be seen, may be compared with leptotene of meiosis

The cytoplasm and nucleus gradually increase in size; the volume of the nucleus is now large enough for the coiled chromosome threads to be clearly seen, fig. 5. At this stage begins the pairing or "somatic synapsis," *i e*, the fusion of the homologous chromosomes (zygotene) and the development of the magma (nuclear meshwork of Painter) The chromosomes fuse, simultaneously their width increases, and the banded structure becomes very clear.

It is most probable that the great increase in volume of cytoplasm and nucleus is correlated with the multiplication of the chromosomes by division of the individual threads two, three, or four times, as in the process found in the octoploid cells of the tracheal tissues In the salivary glands the sister threads remain in close association and compound or multiple chromosomes arise The number of globular particles (chromomeres) at the bands indicates that the division of chromosome fibres is not simultaneous, some of the fibres must have divided more times than others, *cf* Table I.

It may be noted that the failure of the nucleus to divide and of the chromosomes to separate into daughter-chromosomes, as is the rule in an ordinary cell, is associated with the complete filling up of the cytoplasm with the globular bodies, which are apparently the product of its metabolic activity; this, it seems, must necessarily prevent any ordinary process of mitosis

5—Movements of Chromosomes

In the following section the chromosome behaviour is described and discussed from a dynamic point of view. It is necessary to point out that the following observations apply to the uncrushed salivary gland nuclei, which have been studied in order to work out in detail the mechanical changes undergone by the chromosomes during and after pairing.

(*a*) *In Homozygotes*

During the earlier stages of differentiation the single chromosome threads are twisted and coiled in a very densely packed bundle, fig. 35, Plate 25 The coiling, however, can only be followed for a short distance, and not along the whole chromosome In the segments which were suitable for analysis it was found that the coils turn in a constant direction. Later, when the chromosomes are more evenly distributed owing to the

increased space within the nucleus, the coiling of the chromosomes is more apparent, but the analysis of direction of separate coils is still difficult because the twist is very sharp, and it is not easy to see which thread lies on top

The amplitude of coiling is usually the same in successive coils, fig. 23, Plate 23 The coils of the single thread are not evenly distributed; segments with few coils alternate with segments having more numerous coils, fig 5*g* The uneven distribution is most probably the result of smearing, for in slightly pressed preparations it can be seen that the amplitude of separate coils varies very little Great variation was observed in the distribution of coils of different chromosomes within the same nucleus. The variation is due to the external repulsions restricting the uncoiling to a different extent in different portions of the nucleus

The coils of the single chromosome threads are evidently the survivors of those formed at the previous mitotic division, and are analogous to the "relic coils" in ordinary mitosis According to Darlington (1935) an internal or molecular uncoiling takes place during telophase and the external uncoiling always lags behind the molecular uncoiling In the salivary gland, the process of external uncoiling is very slow during the earlier stages, the decrease in number of coils becomes apparent only when the volume of the nucleus is considerably increased There is then a general decrease in the number of coils, which remain evenly distributed

The behaviour of these relic coils in single unpaired chromosomes is difficult to follow in unpressed nuclei because of (1) the thinness of the threads, which only stain slightly, (2) the great lability in the external structure of the chromosome, (3) the limited space within the nucleus, and the compact arrangement of the whole chromosome complement.

It is possible, however, to follow the behaviour of such chromosomes as can be easily distinguished from the others In *D. pseudo-obscura* the X-chromosome, having a median or nearly median attachment constriction, proved to be the most suitable for study It was found that the coiling was evenly distributed in the two arms, and the difference in number of these coils became obvious only at later stages, when the longer arm had more coils than the shorter The direction of coiling is consistently opposite in the two arms and nearly always the coil in the long arm turns to the left and that in the short arm to the right The analysis of direction of relic coils in other chromosomes has shown, furthermore, that the direction is also constant in these chromosomes, two autosomes having right-handed and one a left-handed relic coil The direction of relic coils, as a permanent property of individual

chromosome threads, can therefore be used as a criterion of identification in favourable cases

The number of relic coils in single chromosomes could not be determined accurately In the X-chromosomes there are most probably not more than 16 to 20 in each arm, and this number is reduced slowly to 6–8 before pairing

The pairing of homologous threads begins in the region of the spindle-fibre attachment constriction The single chromosomes come very close together in that segment, and the attachment chromosomes fuse. At this stage the nucleus resembles very closely a polarized zygotene nucleus

The fusion of attachment chromomeres may be explained by assuming an increased attraction at this point in the chromosomes, specific for this chromomere but non-specific (as Darlington suggested, 1932) between different chromosomes It is not improbable that the attachment chromomeres of the multiple threads exhibit this specific force in a much higher degree than in meiosis and that this is comparable with the seemingly unlimited attraction which holds 16 chromomeres together elsewhere and causes the fusion of all attachment chromomeres into one central magma

When the pairing of the homologous chromosomes, which still have some relic coils, begins at the proximal segment, the chromosomes develop a new coiling; and homologues twist round one another This type of coiling is analogous with the relational coiling of chromosomes found at meiosis (Darlington, 1935) At first the association is loose, the threads do not approximate closely, fig 6*c*, but later the association is much closer, fig 6*b* The direction of relational coiling, like that of the relic coiling, is constant in the same chromosome-arm Hence the two arms of the X-chromosome of *D. pseudo-obscura* have relational coiling, the short arm turning to the right and the longer arm to the left, the direction changing at the attachment chromomere embedded in the magma The autosomes, having terminal spindle attachments, show relational coiling in one direction only

The distances between twists vary, there are short and long internodes, fig 6*b*, *c* Later, the distances are more even and usually shorter, indicating an increase in the amount of coiling The fusion of the homologous threads follows their association and takes place in sections, fig 21, Plate 22, fig 26, Plate 23, and fig 6*i* It is very rarely simultaneous in all parts Several instances were found in which the proximal segments had already fused, while the distal parts were left unpaired, fig 6*g*

Intermittent pairing is rare, it is usually a result of some kind of interference at the proximal segment, caused either by relic coils, fig 6*d*, or by interlocking. The free distal parts of homologues very frequently show one or two true relic coils, fig 6*d* But owing to the process of uncoiling of the ends of paired segments by rotation in the development of relational coiling, false relic and relational coils in the opposite direction to that expected were sometimes found at the free distal ends, fig 6, *c*, *e*, *f* The

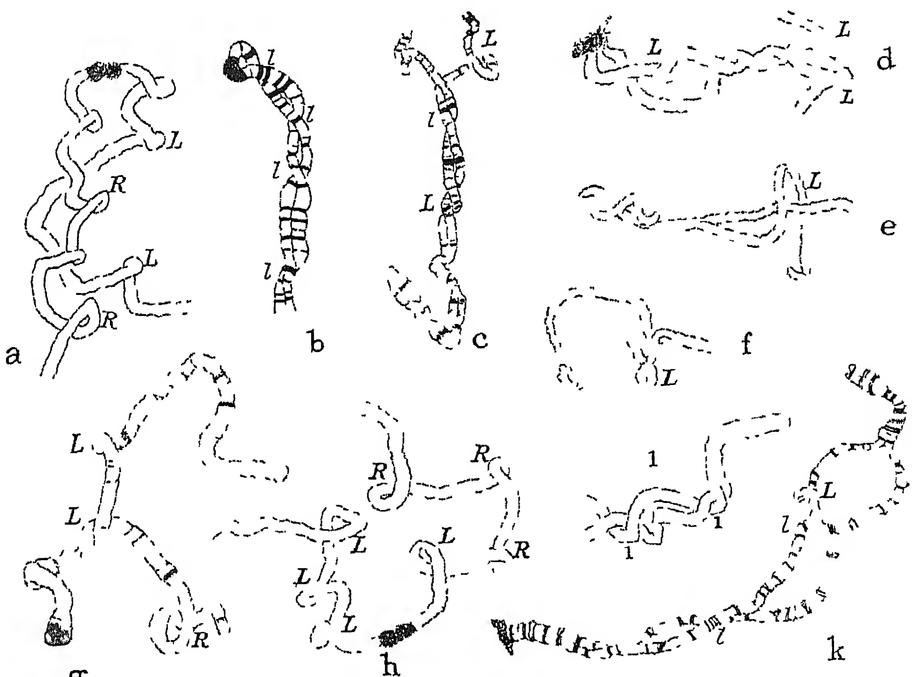


FIG 6—Multiple threads of salivary gland chromosomes showing different types of coiling The direction of coiling is indicated R, right, L, left-handed relic coiling *r*, right, *l*, left-handed relational coiling

number of relational coils varies, in the X-chromosomes of *D pseudo-obscura* it is not more than 16

The number of these relic coils persisting after fusion varies If the fusion occurs with few relational coils, the number of relic coils is 6 to 10, but usually the number is between 4 and 6 in the X-chromosomes of *D. pseudo-obscura.* The distribution of relic coils is even along the fused chromosome, *cf* diagram, fig 7 The direction of coiling can very easily be analysed and it is possible to relate the direction of the other types of coiling in the same individual chromosome with that of the post-fusion relic coils

Changes of direction were found in post-fusion relic coils, fig. 6*i*, and fig. 22, Plate 23, in the arms of the X-chromosome in *D pseudo-obscura*, and are summarized in Table III.

TABLE III—DIRECTION OF RELIC COILING OF X-CHROMOSOME IN 57 NUCLEI SHOWING REDISTRIBUTION (LONG ARM/SHORT ARM)

No of nuclei	L/L	L/L R	L/R	L R/R	R/R	Inconsistent
Certain	1	7	23	4	2	0
Doubtful	4	0	8	2	2	3
Total	5	7	31	6	4	3

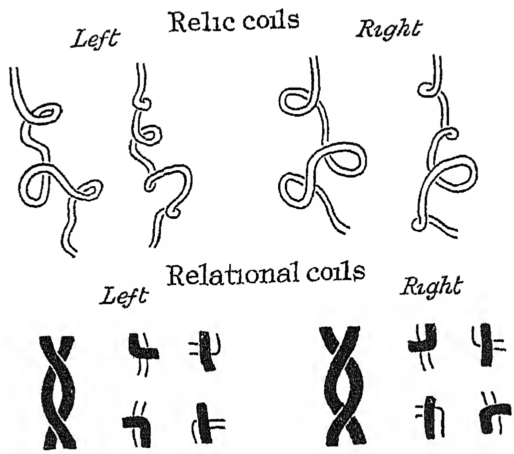


FIG 7—Diagram illustrating the type of relational and post-fusion relic coils as they appear after smearing

Usually the short arm shows right, and the longer left, relic coiling, fig 6*a* The most probable explanation of the changes in direction is that the coiling of one arm becomes transferred to the other arm, as a result of a "redistribution" of coiling strain from the more coiled to the less coiled arm, as occurs in relational coiling at diplotene in meiosis (Darlington, 1935). The loci of changes of direction are proximal rather than distal, as may be seen from the data below *

In the autosomes of *D. pseudo-obscura*, which have terminal attachments, the changes of direction were much less in proportion. Fourteen autosomes out of 77, and 23 sex chromosomes out of 57, show changes in

* In view of the observations in the earlier articles of this series, we need not now suppose, as Darlington (1932, p 290) and Koshy (1933, p 314) have done, that the coiling of the chromosomes must necessarily be in opposite direction in opposite arms, and I do not attach any general significance to this particular observation

direction. The difference of proportion is 0 22 ± 0 01, which means that the arms of sex chromosomes change their direction of coiling significantly more often than the autosomes This is a proof that the latter are independent in their dynamics while the arms of sex chromosomes are connected in this respect

TABLE IV—THE LOCI OF CHANGES IN DIRECTION OF COILING IN THE TWO ARMS OF THE X-CHROMOSOME IN *Drosophila pseudo-obscura*

(INCLUDING PREPARATIONS WHERE ONLY ONE ARM WAS OBSERVED)

	R (short arm)	L (long arm)
Constant coiling	78	64
Proximal change	18	13
Distal change	8	3

The post-fusion relic coils can easily be seen when the multiple threads are released from the nucleus by pressure They retain their coiled structure, figs 24, 25, Plate 23, and resemble the mitotic chromosomes, showing super-spirals at the end of the resting stage (Darlington, 1935)

The internal and external mechanics of chromosomes in the salivary glands described above may be summarized as follows the external uncoiling of chromosomes, which is a consequence of the molecular uncoiling, continues after the resting stage The latter may represent a stage of differentiation of the whole cell The chromosomes divide and multiple threads are formed, containing 2 + 2, 4 + 4 chromosomes The pairing of homologous multiple threads takes place while the external uncoiling is in progress. The pairing is followed by the formation of relational coiling. This type of coiling through the fusion of homologues later becomes invisible, but it persists internally In meiosis the uncoiling of the molecular coiling determines the formation of relational coiling and an internal tension develops which is released only by crossing-over, *cf* Darlington, *loc cit* Such a mechanism does not exist in the chromosomes of the salivary glands; the relational coiling remains side by side with the relic coiling throughout the life of the salivary gland nuclei The nuclei in the salivary gland cells are thus in a state of perpetual prophase corresponding with a modified meiotic prophase, fig 8.

(*b*) *In Structural Hybrids*

The movements of chromosomes and the mechanism of pairing in the presence of homologues showing structural differences must necessarily be a complicated process, and its analysis in the salivary glands, in view of the advantages offered by the great size of their chromosomes, should

throw a better light on the meiotic pairing of structurally different chromosomes

(1) *Intercalary Inversion*—Pairs of chromosomes heterozygous for an intercalary inversion may be associated by complete or incomplete pairing, fig 9 In the latter case, the corresponding segments of chromosomes which show the inversion in their linear arrangement are left unpaired Such unpaired loops, indicating the inverted part of the chromosomes, were very rarely found in completely developed nuclei. In the earlier stages, however, such loops were more frequent, fig 10*a*

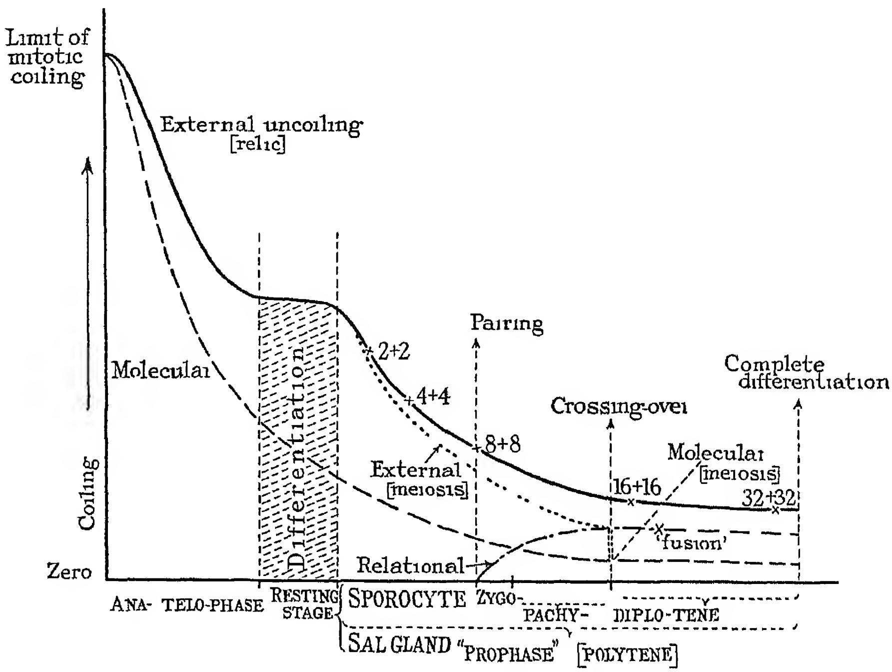


FIG 8—Graph illustrating the movements and the origin of different coiling in the chromosomes of the salivary glands

The single chromosome threads usually have a few true relic coils, and sometimes false ones. The associated segments of chromosomes beyond the inversion are completely fused The non-paired part usually includes the neighbouring distal and proximal homologous segments besides the inverted section, fig 20, Plate 22

I have carefully followed the successive stages by which these inverted segments come into association The formation of a loop is the first step in the process of pairing, fig. 10*a* The next step is the formation of a figure-of-eight, fig 10*b*, by a relational twist, which brings the middle

part of the large loops together The direction of the first twist is usually the same as that of the relational coiling The twisted arms fuse at the point where the overlapping is, and from this centre pairing proceeds towards the distal and proximal breakage points, fig 35, Plate 25. Pairing is accompanied by relational coiling under normal conditions, and such coiling must develop in the associated arms of an inversion, but owing to the fact that the ends of the arms are fixed, *false relational* coiling is necessarily formed on one or both sides of the true coiling, which develops in the parts that pair first to compensate for the true coiling, fig 10, *c*, *d*, *e* The pairing will be hindered and sometimes the middle segments fuse completely, figs 31, 35, Plate 25, before further association

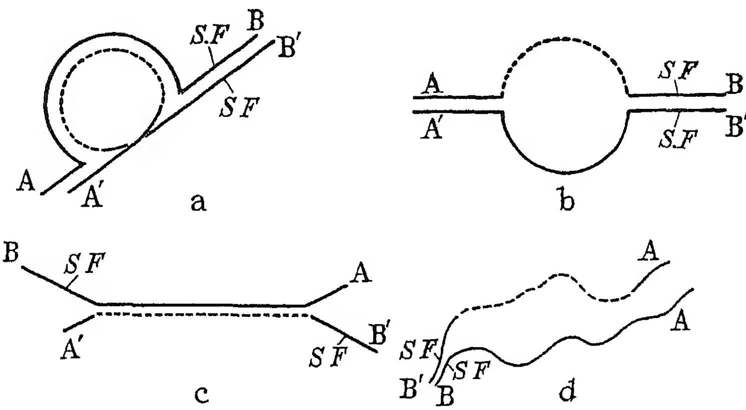


FIG 9—Diagram illustrating. *a*, complete and, *b–d*, incomplete pairing of chromosomes heterozygous for an intercalary inversion S F, spindle fibre or attachment chromomere, A and A′, the distal and B and B′, the proximal homologous segments The inversion is indicated by the dotted line

takes place towards the proximal and distal breakage points of the inversion. Such behaviour explains the genetical results, found in the presence of large inversions, such as those found by Gruneberg (1935) The stage described above in the pairing of inverted segments is very characteristic and apparently of long duration; this seems to show that the development of false coiling delays pairing Sometimes several false relational coils are present, and although fusion proceeds, it occurs sectionally, fig. 10*f*, *g*, *h*, *i*. If the segment distal to the inversion is left unpaired, the inverted segments remain unpaired too The formation of a loop seems to be *conditio sine qua non* of complete pairing in an inverted sense, fig. 11*a* Frequently segments of inverted chromosomes begin to associate at a point distal to the inversion, as shown by the occurrence

of a certain kind of interlocking, fig. 15*c*. If the inversion includes a very large portion of the chromosomes, the loop shows relic coiling like the free parts of the paired chromosomes, fig 17*a*.

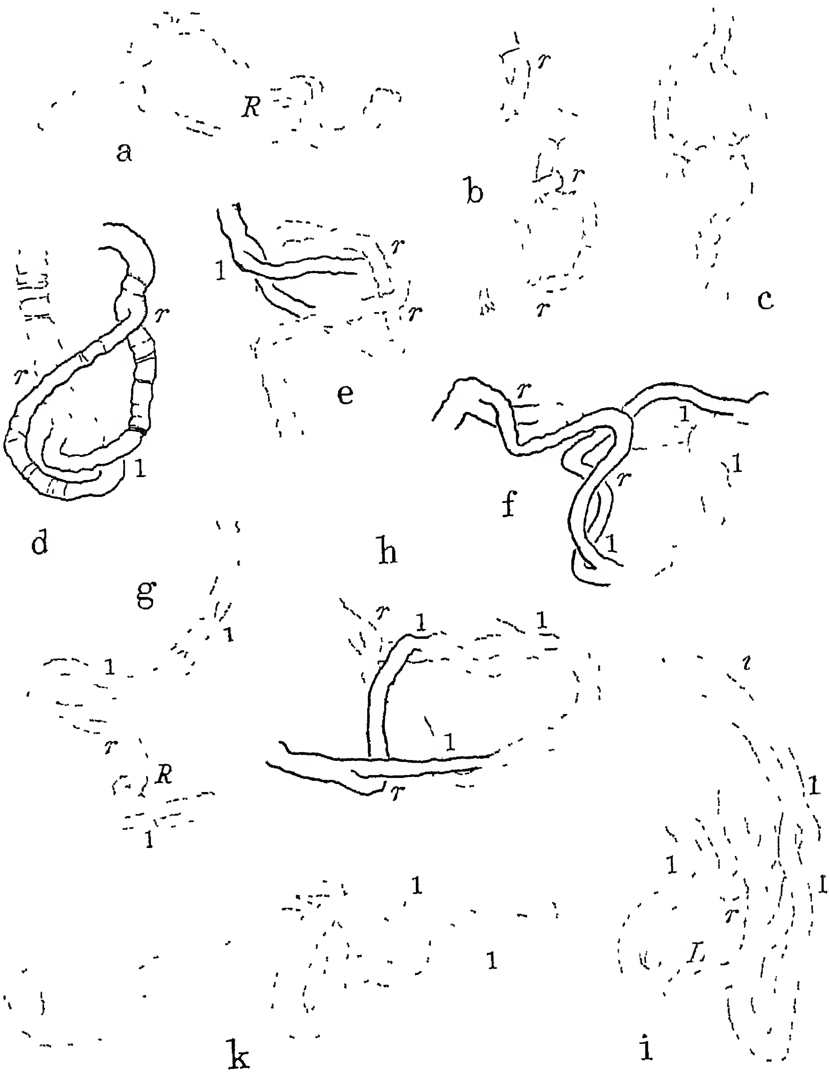


FIG. 10—Multiple threads showing different stages of pairing in the presence of an intercalary inversion

It may be seen from the above description that in the presence of inversion the relational coiling which develops at first promotes the pairing and fusion of the intercalary inversions. But the fixed ends

of the inversion prevent the normal completion of this relational coiling and hinder pairing. Such a disturbance of the mechanism of pairing can be paralleled by the reduction or partial suppression of crossing-over found genetically in chromosomes heterozygous for an inversion At the pachytene stage of meiosis, according to Darlington (1935) a characteristic coiling is present Crossing-over is conditioned by this coiling. Hence the fundamental change in the mechanism of association of chromosomes having inverted segments necessarily leads to a reduction or complete suppression of crossing-over at the ends of the inversion A further and secondary effect of inversion, of course, is the formation of new types of chromosome by single crossing-over in the inversion itself, carrying a duplication or a deficiency, which causes

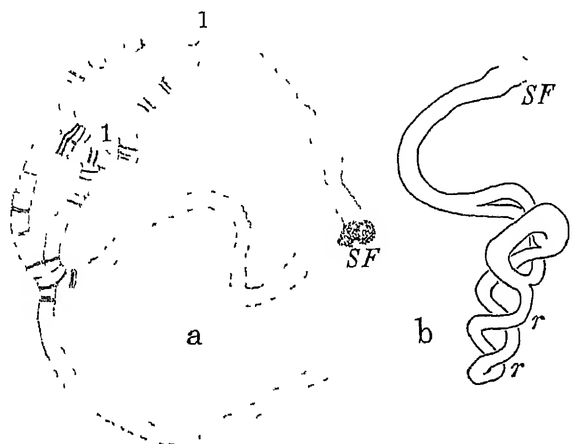


FIG 11—Multiple threads showing the method of association of chromosomes heterozygous for an intercalary inversion

gametic lethality and consequently leads to further reduction of crossing-over.

(ii) *Intercalary Deletion*—A small deficiency in one chromosome of a pair including only three to six bands, 6 to 12 μ in length, was detected by the presence of a small one-sided loop, fig 12*a*, *b*, *c* and fig 30, Plate 24. The pairing on both sides of the deficiency was complete and the homologous threads fused Some instances were found where the threads, one of them the deficient chromosome, showed relational coiling, fig 12*d*, in the region of the deficiency This is proof that the development of relational coiling beyond the deletion is not prevented, and complete association and fusion are rendered possible on both sides of such a small deletion.

When the deficiency involves long segments 30–40 μ in length the loop is larger and very frequently twisted, fig 13*a* and *b*. At earlier stages of development, no sign of such loops indicating deficiencies were seen. the threads, one of them known to be deficient, were associated by loose

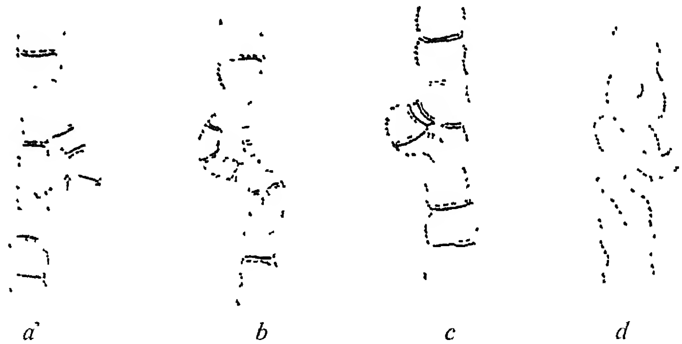


FIG 12—Association of multiple threads heterozygous for *a*, *b*, and *c*, small deletion, *d*, deletion showing relational coiling

relational coiling, distorted to a certain degree in the deficient segment When the association becomes more intimate, and fusion takes place between identical segments, the extra part of the normal chromosome opposite to the deleted thread is left unpaired, fig 13*c*, *d* The internal

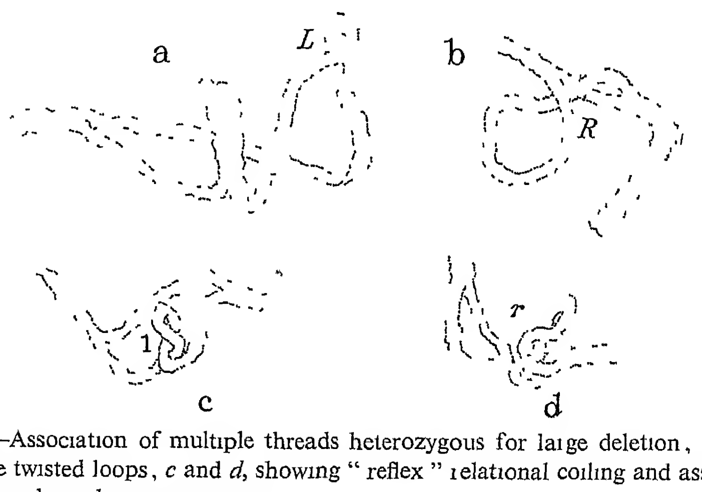


FIG 13—Association of multiple threads heterozygous for large deletion, *a* and *b*, large twisted loops, *c* and *d*, showing "reflex" relational coiling and association on non-homologous segments

uncoiling evidently proceeds in these segments as in the paired regions, and the internal tension necessarily leads to the association of non-homologous parts of chromosomes by relational coiling of the reflexed loop in the characteristic direction (right for the short arm of the X), and

this establishes the necessary equilibrium This "*reflex*" relational coiling will not lead to fusion, it can always be seen even when the homologous segments are fused on both sides of the deletion Pairing of non-homologous segments in deficient chromosomes was described by McClintock (1933) in maize The association of such non-homologous segments by false relational coiling as described above can be seen very clearly in her photomicrographs This association cannot be compared with that found between homologous parts of pairing chromosomes, as was done by McClintock The association of homologous chromosomes is an outcome of the specific, directional attraction, probably chemical in nature, operating between identical constituents

(iii) *Intercalary Translocation*—One geographical race of *D pseudo-obscura* was heterozygous for an intercalary translocation within a chromosome and the pairing mechanism was studied When the two loops caused by the change of homology in linear differentiation are left unpaired, the single threads usually show false relic coils, fig 32, Plate 25, or false relational coiling, as a result of the same internal disequilibrium as is found in loops of deletions

Several cases were found showing complete and incomplete pairing of translocations In about half of the observed cases the loops were left unpaired, in the other half they paired, the proportion of complete to incomplete pairing being 1/2 to 2/3. This is most probably due to the fact that the intercalary translocation involved neighbour segments, and the rigidity of the chromosome rendered it more difficult than in translocations between non-homologous chromosomes

The direction of relational coiling was inconsistent, fig 14 The non-paired threads are usually drawn out, giving asymmetrical configurations By crossing-over in the translocation, deficiencies may arise and increase the structural differences The pairing under such conditions is further hindered and leads to more complications in the whole mechanism, fig 14*b*, *b*′, and fig 30, Plate 24, showing translocation combined with deletion The reduction of crossing-over at meiosis is due not only to the decreased pairing possibilities but most probably to the disturbances in the system of coiling in the same way as was discussed for inversions.

6—Interlocking and other Abnormalities

In the nuclei of the salivary glands chromosome interlocking, like that at meiosis, was very frequently seen. The most probable causes of the frequent interlocking are (1) the great length of the chromosomes,

(2) the relatively limited space within the nucleus, and (3) the general movements of the chromosomes, *i e.*, the uncoiling of the relic coils and pairing by the formation of relational coiling

The interlocking may be "false" or "true," and either may be "external" or "internal" (*cf* Darlington, 1932) The different types are illustrated, fig 15 The most common type of false interlocking is when a paired multiple thread is included in the loop of a relic coil of another chromosome, fig 15*a*. It may prevent a free distribution of the

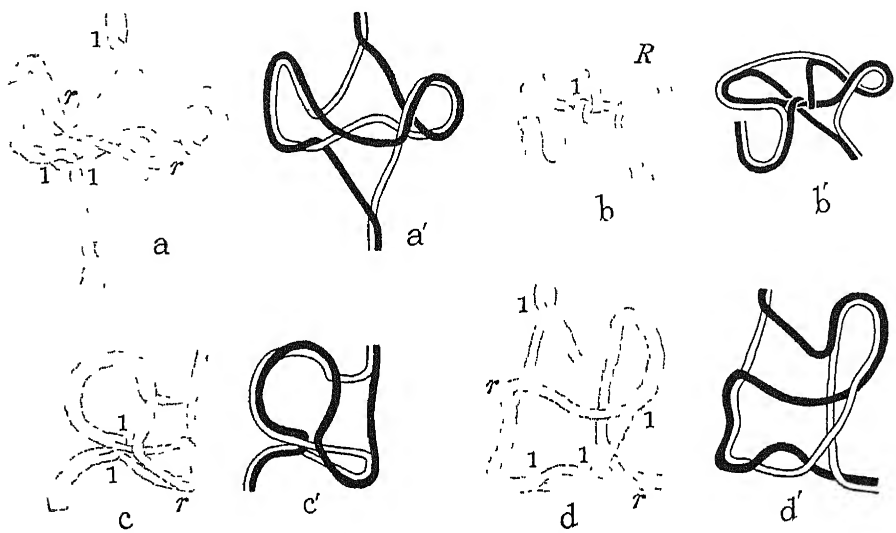


FIG 14—Camera lucida drawings and diagrams of multiple threads showing the method of pairing of homologues heterozygous for *a*, *c*, and *d*, intercalary translocation within the chromosome, *b*, the same translocation combined with deletion

relic coils, and preserve the number of such coils in proximal segments

Another type of false interlocking was found commonly in inversions when one chromosome was inserted into a loop of the inversion, fig 15*b*. The result is usually a delay in pairing of the homologous segments and development of a false relational coiling False internal interlocking was found in an inversion, when the loop was caught between the single arms, fig. 15*c*, and fig. 34, Plate 25. It is possible that the loop would later be released by a twist This type of interlocking proves that pairing proceeds from the middle part of the inversion towards the ends Another false internal interlocking is illustrated in fig 15*f*. The same chromosome prevents the pairing of its homologous members further along the thread.

This will disarrange the normal relational coiling and delay or prevent fusion in a long section

True external interlocking, with two non-homologous chromosome pairs, is a comparatively rare phenomenon It prevents pairing in both pairs, fig 15*g* This proves that the association of homologous threads may be intermittent The non-paired intercalary segment does not prevent the complete pairing of the segment distal to the unpaired section The relational coiling develops normally, the cause of its development and the mechanism being identical with those operating in the segment proximal to the unpaired section.

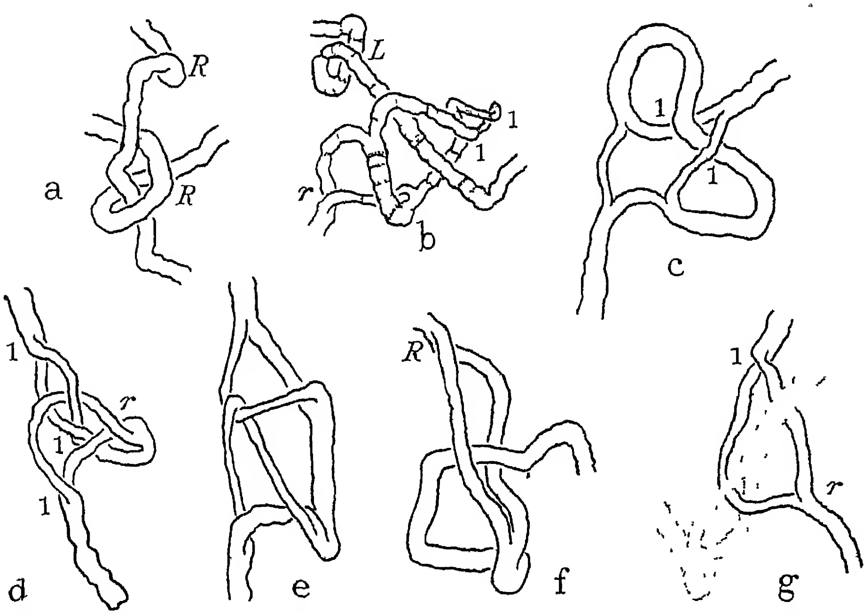


Fig. 15—Chromosomes showing different types of interlocking The direction of coiling is given

Two types of true internal interlocking were found One of them is illustrated in fig. 15*d* and is similar to those described by Gelei (1921) in *Dendrocoelum* and Koller (1935, *b*) in marsupials The same two homologous chromosomes are prevented from pairing in two intercalary segments, separated by a paired segment

The other special type of true internal interlocking requires a more detailed analysis In this kind of interlocking, fig. 15*e*, and fig 16, the "reflex" relational coiling—just the same kind as was described in the presence of deletion—was developed and caught between two paired segments in a homozygote Different stages in this interlocking were

found in nuclei of salivary glands, and cases observed in the short arm of the X-chromosome are shown in fig 16A–C, and fig 37, Plate 25 The large intercalary segment was delayed in pairing It formed a loop, and reflex relational coiling developed in one of the single threads In the following stage, the homologous middle parts paired. The number of the reflex relational coils was 2, 4, or 6, *i e*, always even, which is necessary for pairing in the same direction As the pairing and fusion proceeded, the reflex relational coiling was restricted though not eliminated, since uncoiling was prevented by the distal and proximal sections being fixed The single arms were drawn out, showing the tension caused by the pairing further along the homologous chromosomes It seems possible that the tension in a longitudinal direction might be very great, and the single arms (*a*, *b*, *c*, and *d* in fig 16C) drawn out so much that they would break A subsequent fusion of the broken arms would

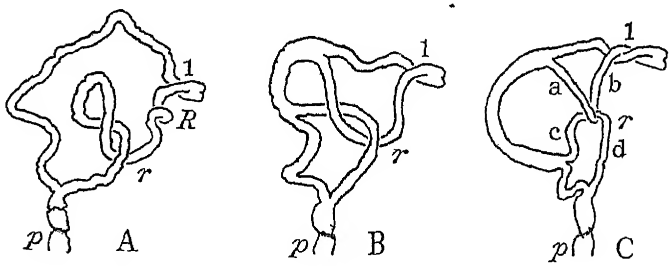


FIG 16—Camera lucida drawings illustrating different stages of true internal interlocking, *cf* fig 37, Plate 25

lead either to inversion (if *a* fuses with *d*, and *b* with *c*) or to deletion (if *a* fuses with *c* and *b* with *d*) Such a configuration at meiosis would therefore explain the origin of inversion and deletion, the ultimate cause being the reflex relational coiling following intermittent pairing

7—THE UNDIFFERENTIATED MAGMA

The nuclei of salivary glands during the earlier stages of differentiation contain single threads which radiate from a centre; the whole configuration closely resembles polarization, as already mentioned. Later in development it was found that it is the proximal segments which come into close contact, and such a chromosome arrangement was assumed to be due to the secondary pairing operating at the previous mitotic metaphase

The Y-chromosome and the genetically inert region of the X-chromosome do not appear to be represented in the nuclei of salivary glands

(Painter, 1934) It is certain, however, that these elements are present—although invisible—in the nuclei When the prophase begins, the Y-chromosome is most probably left at the locus—called the pole—which was occupied by this element at telophase and the resting stage During the first stages of differentiation, the "polarization" is obscured by the presence of a large, very lightly stained mass, which represents the Y and the inert region of the X, fig 28, Plate 24 The condensation of this centre, however, is rapid and simultaneous with the fusion of the attachment chromomeres The two arms of X in *D pseudo-obscura* could not be identified at the very early stages of differentiation, because the inert segment on both sides of the attachment chromomere was left undifferentiated, and the arms were apparently not connected When the condensation of this undifferentiated region is completed, the two arms are brought closely together at the attachment chromomere, fig 35, Plate 25

Besides the complex of fused attachment chromomeres there is usually present a slightly stained mass which does not show any structure in the living condition. Very frequently it is connected with the complex of attachment chromomeres and makes the analysis of the extreme proximal segments difficult, fig 33, Plate 25 This mass, the nuclear meshwork of Painter, is termed (following the suggestion of my colleague Darlington) the *magma*. The function of the magma is at present unknown, and no satisfactory explanation can be given for the behaviour of the Y- and part of the X-chromosome

The inversion found by Gruneberg (1935) in the X-chromosome of *D. melanogaster* includes a portion of the genetically inert region The study of this inversion makes it possible to get a better insight into the behaviour of the inert region in the salivary glands The change in linear arrangement occurred in the inert region and beyond *ruby*, fig. 17*a*, but the intercalary position of the inert part does not prevent the pairing It suggests that the connection between segments separated by the inert region is not severed in the salivary glands Usually condensation takes place, and as a result of it a large, structureless mass will be formed at the point containing the attachment chromomere, the inert region and the loci of breakage, fig 17*b*, *c*

Other evidence was found, which further suggests that the inverted segment does not interrupt the internal relationship between the two arms of the X-chromosome in *D. pseudo-obscura* by the process of redistribution of relic coils from one arm to the other The statistical analysis demonstrates that the changes in the direction of relic coils are more frequent in the X-chromosomes than in the autosomes and that

they are always proximal—adjoining the arm of opposite coiling habit. The latter, which have terminal attachment chromomeres, so that one individual chromosome is represented by a single arm in the nuclei of the salivary glands, are more independent from one another in their behaviour. The two arms of the X are therefore a mechanical unit.

The attraction between attachment chromomeres is not only non-specific, as perhaps occurs elsewhere (*cf.* Darlington, 1932, on *Agapanthus*), but, like

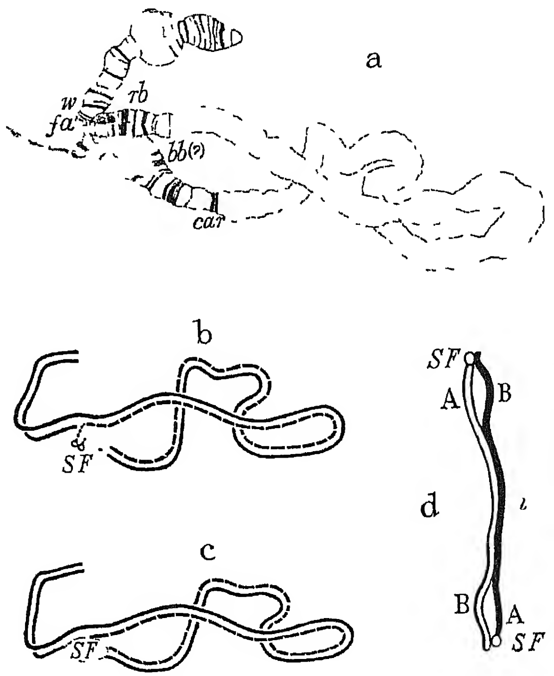


FIG. 17—*a*, X-chromosome of *D. melanogaster* heterozygous for an intercalary inversion which includes a part of the inert region; *b* and *c*, diagrams showing the same inversion; *d*, diagram illustrating incomplete pairing of multiple threads heterozygous for a large intercalary inversion. The attachment chromomere S F fuses with the non-homologous end of other chromosomes, *cf.* fig. 29, Plate 24.

the other attractions, unlimited, so that the attachment chromomeres of all chromosomes, homologous and non-homologous, fuse into one body. Sometimes, however, the attachment chromomere fuses with another part of the chromosome, as was found in incomplete pairing of a large intercalary inversion, fig. 17*d*, and fig. 33, Plate 25.

Sometimes two centres of fusion were seen in the nuclei of the salivary glands. One of them contained the two arms of the X-chromosome and one autosome, fig. 20, Plate 22, and fig. 30, Plate 24, while the other included

two long autosomes and the dot-chromosome. This condition was more frequent in young nuclei, and at the time of complete differentiation the number of centres was usually one

I am greatly indebted to the Trustees of the John Innes Horticultural Institution, London, for a grant which made it possible for me to complete this work, and also to Dr C D Darlington for his help and criticism.

8—Summary

The banded chromosomes of the salivary glands of Diptera consist of 4–8–16 chromosomes made up of the characteristic chromomeres lying side by side.

Homologous multiple chromosomes pair side by side and fuse into one cylinder

These multiple chromosomes show relic coiling before pairing, and both relic and relational coiling after pairing The direction of this coiling is characteristic of the particular chromosome arms.

The torsion which determines the relational coiling also leads to reflex relational coiling within chromosomes, especially in redundant segments (opposite deletions).

The pairing of inverted segments is promoted by the development of relational coiling between them

The relic coils decrease in number and increase in amplitude as development proceeds. Occasionally, the type of coiling of one arm is " redistributed " to another arm of the X-chromosome which has a characteristically opposite direction of coiling

These properties of coiling are analogous with those found in the prophases of mitosis and meiosis, and are presumably due to analogous changes in the molecular spiral, whose coiling determines the spiralization of metaphase chromosomes

9—References

Balbiani, E G (1881) 'Zool Anz' vol 4, pp 637, 662
Bridges, C (1935). 'Amer. Nat,' vol 69, p 59
Darlington, C. D (1932, *a*) 'Cytologia,' vol 4, p 229
—— (1932, *b*) " Recent Advances in Cytology," London
—— (1935) 'Proc Roy Soc,' B, vol 118, p 33
Gelei, J (1921) 'Arch Zellforsch,' vol 16, p 88.
Grüneberg, H. (1935) 'J. Genet' (*in the press*)
Heitz, E., and Bauer, H (1933) 'Z Zellforsch u Mikr Anat,' vol. 17, p 67.

Koller, P C (1935, *a*) 'Nature,' vol **135**, p 69
—— (1935, *b*) 'Cytologia,' (*in the press*)
Koltzoff, N (1934) 'Science,' vol **80**, p 312
Koshy, T K (1933) 'J. Roy Micr Soc,' vol. **53**, p 300
McClintock, B (1933) 'Z Zellforsch u. Mikr Anat,' vol **19**, p 191.
Muller, H J, and Prokofyeva, A A (1935) 'Proc Nat Acad. Sci, Wash,' vol **21**, p 16
Painter, T S (1933) 'Science,' vol **78**, p 585
—— (1934) 'Genetics,' vol **19**, p 448

Description of Plates

Plate 22

Fig 18—Segment of a chromosome or multiple thread in the salivary gland of *Chironomus* showing numerous fibres

Fig 19—Nucleus of salivary gland in *Drosophila* The individual bands show numerous granules or chromomeres *a*

Fig 20—Three multiple threads are fused at the attachment chromomere S F The crenate outline of the bands can be seen One thread is heterozygous for an intercalary inversion, the homologues do not pair for a long section beyond the inversion

Fig 21—Fusion of two homologous chromosomes, the identical arrangement *a* of bands and the relational coiling can be seen

Plate 23

Fig 22—Chromosome showing changes in direction of relic coils. R, right, L, left-handed turn The relational coiling is right handed, *b*, and false interlocking *a* is formed

Fig 23—Chromosome showing pre-fusion relic coils

Figs 24 and 25—Post-fusion relic coils partly released before fixation

Fig 26—The fusion of homologous chromosomes showing right-handed relational coiling

Plate 24

Fig 27—Complete nucleus of salivary gland The chromosomes show the post-fusion relic coils

Fig 28—The attachment chromomere S F. (X) between two arms of the X-chromosome in *D pseudo-obscura* The other portion of the magma M can be seen separately

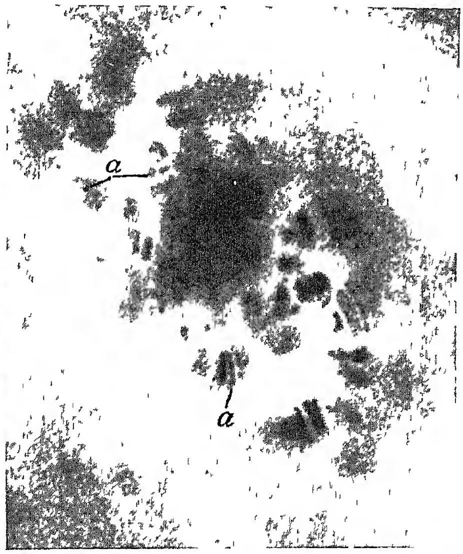
Fig 29—Complete nucleus, the chromosomes show relic coils One thread is heterozygous for a large intercalary inversion and the homologous chromosomes are incompletely associated at *a* and *a'*, *cf* fig 9*c*

Fig 30—Chromosomes showing *a* partial association, *b* small deletion, and *c* intercalary translocation combined with deletion, *cf* fig 14*b* and *b'*

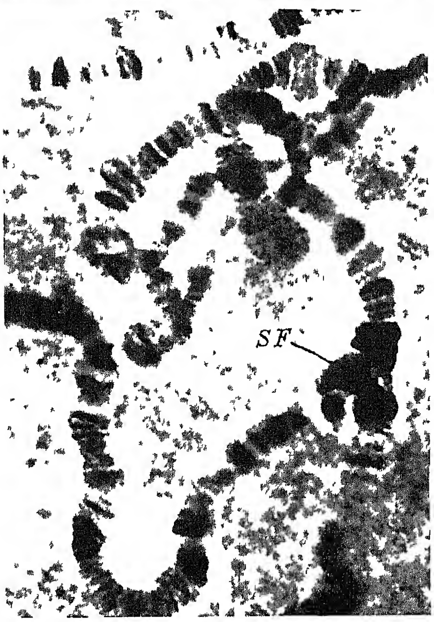
Fig 36—Salivary gland cell, showing the chromosomes densely packed in the nucleus, *cf*. fig. 5

18

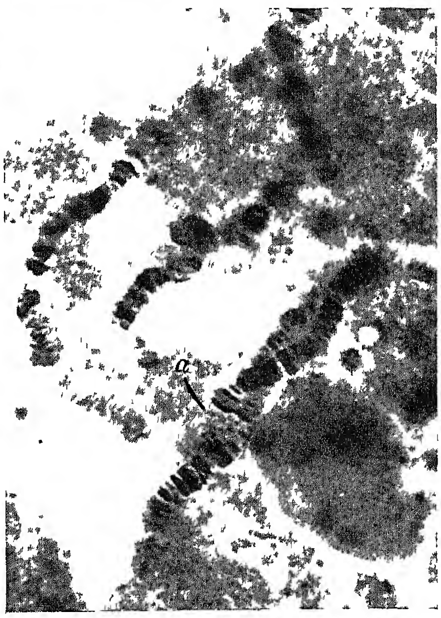
19



20

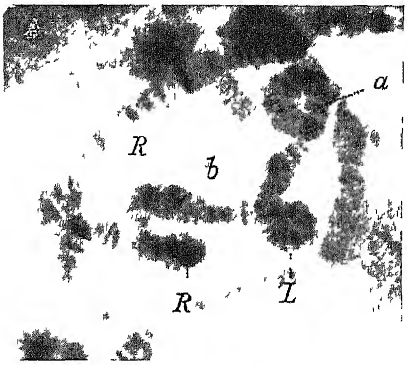


21



(*Facing p* 396)

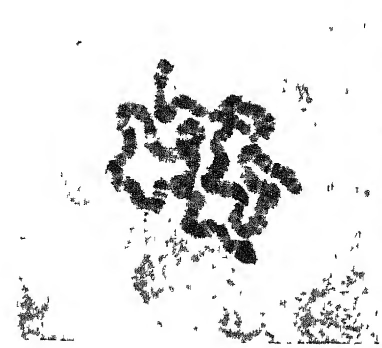
22



23

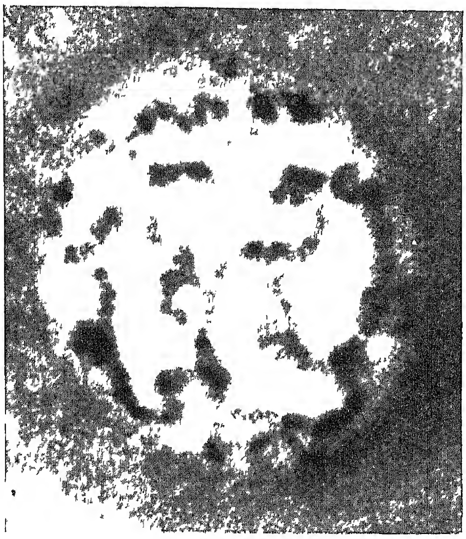
24

25

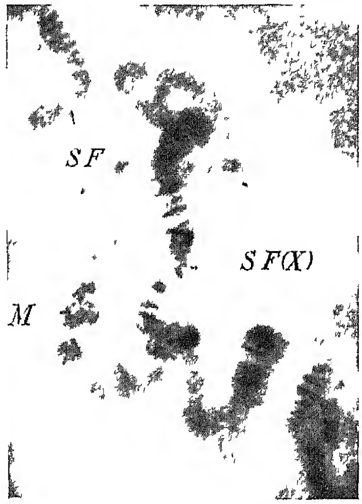


26

27



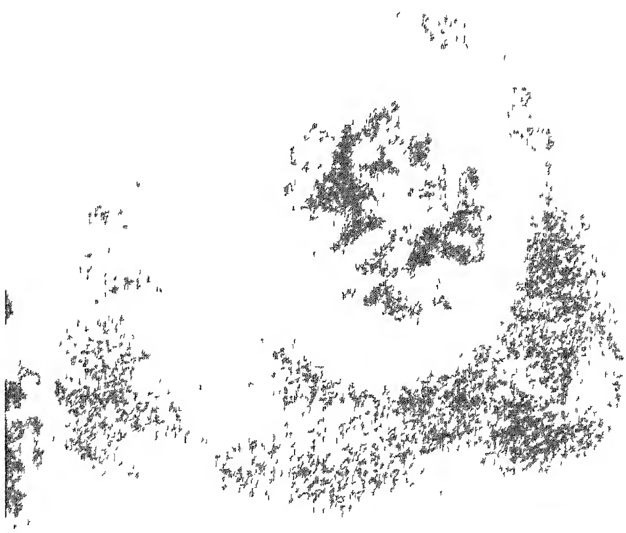
28



29

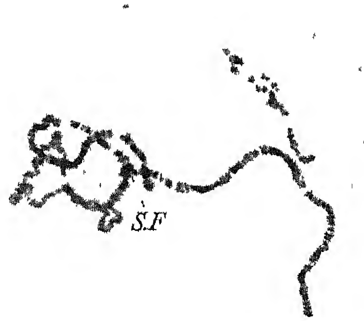
30

36



31

32



33

34

35

37

PLATE 25

FIGS 31 and 33—Pairing of chromosomes heterozygous for an intercalary inversion. Fig 31—only the middle section of the inversion loop is fused, fig 33—more complete pairing in the inverted segments

FIG. 32—Chromosome carrying an intercalary translocation within a chromosome. The unpaired segments of homologues show pre-fusion relic coils

FIG 34—Interlocking of an inversion loop, *cf* fig 15*c*

FIG 35—The X-chromosome of *D pseudo-obscura*, one arm is heterozygous for an intercalary inversion, which shows incomplete pairing

FIG 37—Microphotograph showing true internal interlocking, *cf* fig 16

---

612 21 595 775

The Regulation of Respiration in the Flea, *Xenopsylla Cheopis*, Roths. (Pulicidae)

By V B WIGGLESWORTH, M A., M D

From the London School of Hygiene and Tropical Medicine

(*Communicated by J. S Haldane, F R S—Received July* 3, 1935)

INTRODUCTION

The gaseous exchanges of terrestrial insects are regulated by (i) the opening and closing of the spiracles—the "diffusion control" of Hazelhoff (1926, 1927)—and, in the larger and more active forms by (ii) the mechanical aeration of the tracheal system by pumping movements—"ventilation control" Spiracular and pumping movements may occur at the same time, for example, in those insects in which a directed stream of air is driven through the main tracheal branches (Fraenkel, 1932, McGovran, 1931); and then the spiracles, by allowing air to pass through them in one direction only, are believed to play a part in the mechanism of ventilation

The object of the present work was to study in greater detail than hitherto the spiracular movements of an insect in which these are not complicated by mechanical ventilation For this purpose the common rat flea of the tropics, *Xenopsylla cheopis*, has proved an ideal subject.

ANATOMY OF THE RESPIRATORY SYSTEM

Fig. 1 shows the arrangement of the main tracheae in the flea, and the position of the spiracles. There are three thoracic and seven abdominal

spiracles on either side of the body. The prothoracic spiracle, Th I, opens at the apex of a minute papilla behind the base of the propleuron, the mesothoracic, Th II, on a similar papilla behind and below the mesopleuron The metathoracic, Th III, is a large spiracle placed rather high up on the back, close to the upper margin of the metepimeron The first abdominal segment has no spiracle, in the six following segments Abd ii–vii, the spiracle lies at the side of the abdominal tergites; while the spiracle of the eighth segment, Abd viii, opens dorsally on the eighth tergite and is connected with a deep corrugated groove which extends almost to the mid-line This last abdominal spiracle is larger than the others and leads to a larger trachea

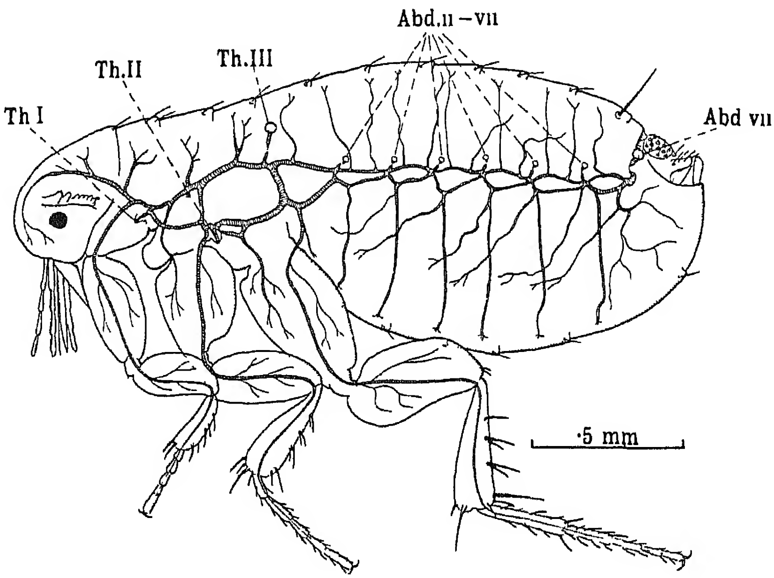


Fig. 1—Tracheal system of *Xenopsylla cheopis* Th I–III, thoracic spiracles, Abd ii–viii, abdominal spiracles

All the spiracles have a closing mechanism In Th III and Abd ii–viii, near the external opening, the trachea narrows to about half its diameter for a short distance. At this narrow point the spiral folding is wanting and the trachea is crossed by a little crescentic rod of chitin; it actually lies in a deep groove in the concave surface of this rod. The two ends of the rod are joined by a muscular strand, when this contracts the trachea is pinched and occluded,* fig 2, A–D.

* This mechanism was briefly described by Landois and Thelen (1867) and Krancher (1881)

In Th I and II, the mechanism is quite different Here the little papilla on which the spiracle opens has its apex covered by soft flexible cuticle But from its rigid wall there arises a minute muscle which is inserted into the trachea near its opening, fig 2, E When this muscle contracts the trachea is drawn inwards and the soft tip of the papilla occludes the external opening, fig 2, F, G *

The opening and closing of the sphincters in the abdominal and metathoracic spiracles is readily seen by transmitted light in the living flea lightly compressed beneath a coverslip The erection and retraction of

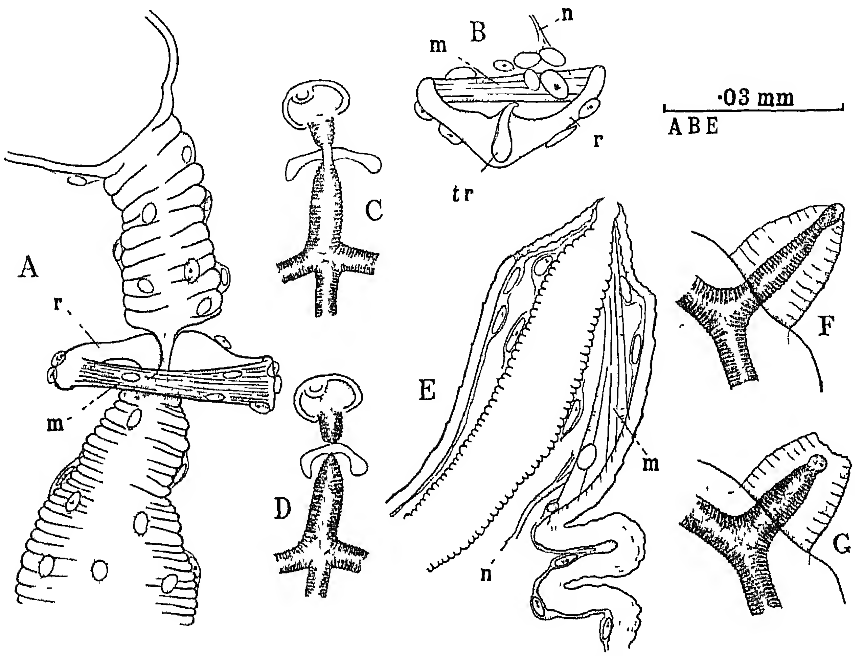


Fig 2—A, Abd viii in surface view, B, Abd vi in transverse section, C, Abd vi as seen in living insect open, D, the same, closed, E, Th II in horizontal section, F, Th II as seen in living insect, open, G, the same, closed *m*, muscle, *n*, nerve, *r*, chitinous rod, *tr*, trachea

the papilla on the mesothorax is very easily seen under the same conditions, that on the prothorax with rather more difficulty

Methods

The methods used do not differ in principle from those employed by Hazelhoff (1926, 1927) in studying the spiracles of the cockroach The

* I am indebted to Dr M. Sharif for calling my attention to a description of this mechanism by Wagner (1889)

flea is enclosed in the gas chamber previously described (Wigglesworth, 1930) and held in place by light pressure between the coverslip and the glass stage Before entering the chamber the gas passes through a two-way tap, by turning which it can be instantly changed Ordinarily, one arm of this tap is connected to the compressed air supply, the other to a gas mixture of known composition in an aspiratory bottle over paraffin, the composition of the mixture being checked with a Haldane gas analysis apparatus

The various observations are recorded on a revolving drum by means of electrical signal markers Three switches are used two tapping keys manipulated by the feet, and a du Bois Reymond key worked by hand In nearly all the tracings which follow, the top line represents the change in gas mixture, the second line records active muscular movements (struggling) by the flea, and the bottom line the opening (downwards) and closing of the spiracle in question The records do not take into account the *degree* of opening in the spiracle, when there are significant differences in this respect they will be mentioned in the text

Movement of the Spiracles in Air

Like the insects studied by Hazelhoff (1926, 1927), the flea at rest keeps its spiracles mostly closed; opening them from time to time to allow the gaseous exchanges to take place When the metabolism is increased (i) during the digestion of blood and the maturation of the ovaries, (ii) by a rise in temperature, or (iii) by muscular activity, the spiracles are opened more frequently.

The movements of Th I and II are determined mainly by the muscular contractions in the legs and thorax Even in fleas at the height of digestion of a large meal, in which the remaining spiracles are open nearly all the time, Th I and II may stay permanently closed except when the legs are moved, fig. 3, A They always open *after* the leg movements have begun, sometimes even after they have ceased. Obviously the opening is determined chemically, as was demonstrated by Hazelhoff in the cockroach, and is not due to a nervous stimulus coincident with the onset of muscular activity as seemed to be the case in the insects studied by Fraenkel (1932). In some fleas, on the other hand, particularly if the temperature is high. there may be a rhythmical opening and closing of Th I and II independent of exertion, though exaggerated when leg movements do occur, fig 3, B

Th. III—In the unfed flea at 20°–22° may remain closed except after muscular contractions in the thorax, fig. 3, C. More often it shows a

rhythmical opening and closing, fig. 3, D When the stomach contains blood, this spiracle is nearly always permanently open

Abd ii–vii—Are generally closed in the unfed flea, and remain so in spite of active muscular contractions, fig 3, E After a meal, when the stomach is in constant peristaltic movement, they open and close rhythmically, the opening being generally rather narrow, fig 3, F The movements of adjacent spiracles are then practically synchronous In female fleas with the abdomen filled with developing eggs and the stomach distended with blood, they remain widely open the whole time

Abd viii may open very rarely in the fasting flea, or only after muscular exertion, fig 3, G. But usually, like Th III, it shows a rhythmical movement which, if the flea does not struggle, may continue indefinitely

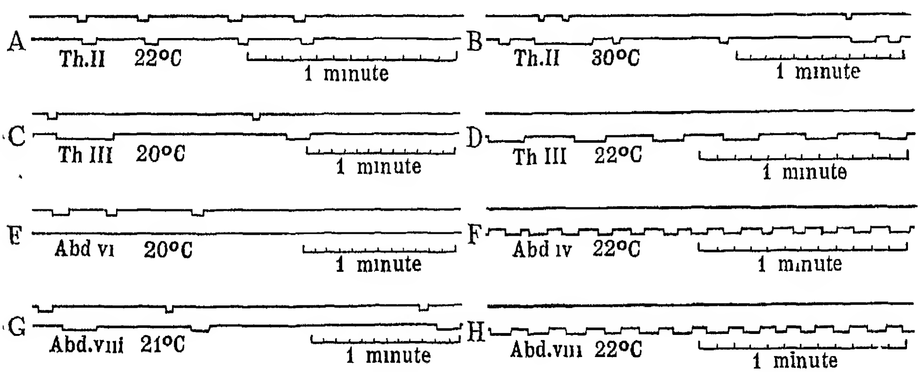


FIG 3—Movements of spiracles in air Upper line of each tracing shows periods of struggling, lower line shows opening (downwards) and closing of the spiracles. The physiological state of the insects used is given in the text

with perfect regularity, fig 3, H When eggs are being developed, Abd. viii remains widely dilated

Thus the two most important spiracles are the two largest ones. Th. III and Abd viii These are nearly always functional, even when the others are permanently closed

Any of the spiracles may be used for testing the effects of different factors upon their movements, but in order to detect very slight effects it is necessary to observe a spiracle showing a regular rhythm, for this reason, in most of the experiments to be described, Abd viii has been used

EFFECT OF OXYGEN ON THE SPIRACULAR MOVEMENTS

The chief problem in the physiology of respiration in the flea is the nature of the spiracular rhythm, nothing like which has previously been

observed in insects. It may be said at once that no evidence has been obtained to suggest that it is due to a spontaneous rhythmic discharge of impulses from the nerve centres All the evidence points to each act of opening or closing being determined by an immediate stimulus of a chemical nature upon the respiratory centres Does this stimulus arise

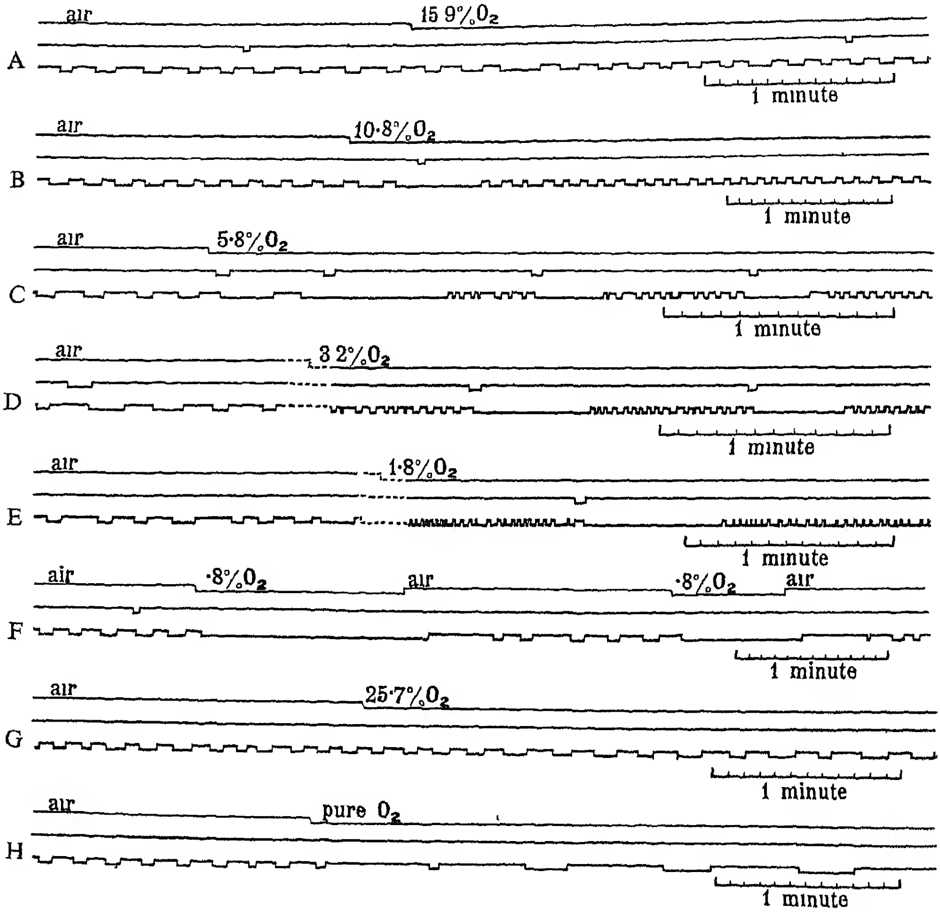


FIG 4—Effect of different concentrations of oxygen in nitrogen upon movements of last abdominal spiracle, *Abd* VIII. Note the prolonged opening caused by struggling in C, D, and E All at 20–22° C

from lack of oxygen or excess of carbon dioxide? According to the view that will be put forward, *both* these factors are concerned—though each has a slightly different influence.

Fig. 4 shows the effect of changing the partial pressure of oxygen to which the flea is exposed. Decreasing the oxygen causes an acceleration of the spiracular rhythm, which becomes progressively more rapid at

15·9, 10·8, 5·8, and 3·2% of oxygen, while at 1·8% it is almost impossible to record the movements accurately. Below 1% of oxygen the spiracle remains permanently open; but on readmission of air it closes again within a few seconds and the original rhythm is restored, fig. 4, F. At these very low concentrations *all* the spiracles are affected and remain widely dilated.

At partial pressures of oxygen above the normal, the rhythm is retarded—slightly at 25·7%, fig. 4, G, greatly in pure oxygen, fig. 4, H.

Some information about the control of the spiracular movements can be obtained by studying these changes in rhythm more closely. The rhythm consists of alternating periods during which the spiracle is open or closed. We have seen (p. 401) that the movement of the spiracles is more or less synchronous. Therefore, during the closed period the partial pressure of oxygen in the tracheal system must be gradually falling, and the partial pressure of carbon dioxide rising. If the carbon dioxide were solely responsible for the regulation, the duration of the closed period should not be affected by the partial pressure of oxygen. But we have seen that this period becomes shorter as the oxygen becomes less, which suggests that the closed period is brought to an end by oxygen want.

If this were entirely true we should expect the duration of the closed period to be proportional to the oxygen content (assuming that the oxygen consumption of the insect remains unchanged). Table I, No. 1–5, shows that this is approximately true at all the oxygen concentrations tested. But in Table I, No. 6, an insect with a rather high rate of metabolism (as evidenced by the short closed period), the degree of lengthening of the closed period on going from air to pure oxygen is considerably less than the calculated figure, which suggests that here the carbon dioxide accumulating during the closed period is itself contributing to the effective stimulus.

But the open period also is affected by changes in oxygen in the same sense as the closed period. Whereas, if the duration of this period were determined by the time taken for the relief of oxygen want, we should expect it to be altered in the opposite sense: to be longer when the concentration of oxygen is low. The duration of the open period is, in fact, more or less proportional to the duration of the closed period which preceded it. Now throughout the closed period, carbon dioxide must be accumulating in the tracheal system and in the tissues. When the spiracles open, more carbon dioxide will be produced during the relief of the oxygen want. It seems likely, therefore, that the duration of the open period may be determined by the time taken for carbon dioxide to diffuse

away. Hence the great length of this period in pure oxygen, where much carbon dioxide will be formed before the insect suffers from oxygen want

But, on the whole, changes in the concentration of oxygen have a greater effect upon the duration of the closed period than upon the open period (*see* Table I)

TABLE I

No of insect	O_2 %	Mean duration of closed period secs	Percentage change in closed period: Observed	Percentage change in closed period: Calculated*	Mean duration of open period secs	Percentage change in open period
1	20 9 15 9	8 0 5 5	31 2	(24 0)	5 1 4 5	11 8
2	20 9 10 8	5 2 2 2	57 7	(48 4)	7 0 4 4	37 4
3	20 9 5 8	7 2 1 7	76 4	(72 3)	6 0 1 8	70 0
4	20 9 25 7	5 8 7 8	34 5	(23 0)	5 4 6 8	25 9
5	20 9 100	7 0 29 3	317 8	(379 3)	4 2 15 5	269 0
6	20 9 100	5 2 17 3	233 3	(379 3)	7 0 17 9	156 0

* Calculated on the assumption that the length of the closed period is proportional to the oxygen percentage

EFFECT OF CARBON DIOXIDE ON THE SPIRACULAR MOVEMENTS

At low concentrations the effect of carbon dioxide varies greatly with the intensity of metabolism Thus 1 4% has scarcely any effect on quiescent fasting insects in which the spiracles are closed most of the time, but causes the spiracles to remain permanently open in insects at the height of digestion The lowest concentration which causes persistent opening of all the spiracles, at 20° C, even in fasting fleas, is about 2%, fig 5, B, a figure which agrees very well with that found by Hazelhoff (1926) for the cockroach

Low concentrations of carbon dioxide, such as 0 93%, fig 5, A, though they fail to cause permanent opening, bring about a constant change in the spiracular rhythm. the closed periods are slightly shortened, but the open periods are much more definitely prolonged Table II shows results obtained with this gas mixture on two fleas Here the closed period was shortened by 8–10%, while the open period was lengthened by 30–50%.

TABLE II

Flea	Gas	Mean duration of closed period secs	Mean duration of open period secs	Percentage shortening of closed period	Percentage lengthening of open period
1	Air	8 3	7 7	8 4	50 6
	0 93% CO_2	7 6	11 6		
2	Air	8 4	8 2	10 7	30 5
	0 93% CO_2	7 5	10 7		

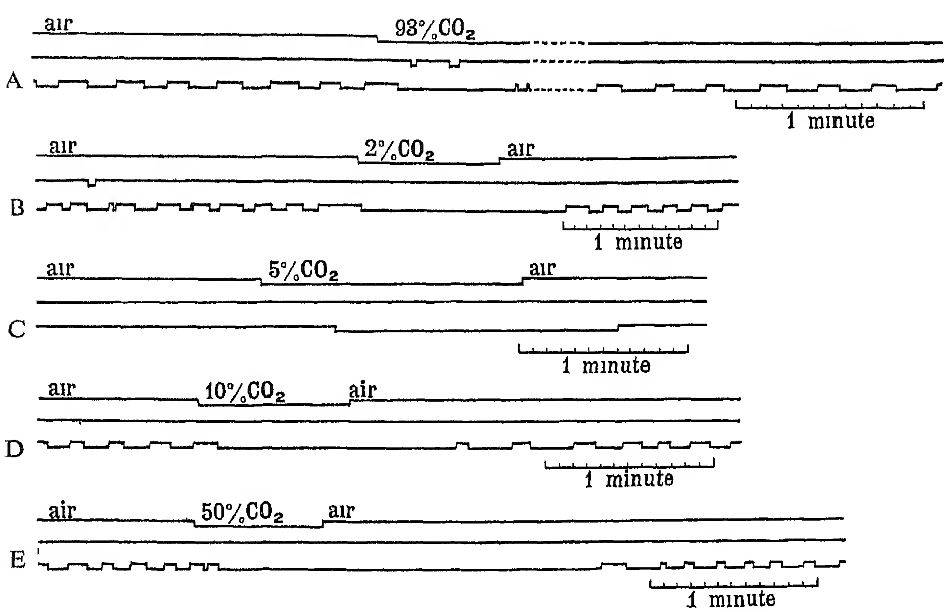


FIG 5—Effect of different concentrations of carbon dioxide in air upon movements of spiracles. C represents Abd vi, the remainder represent Abd. viii All at 20–22° C

These results support the conclusions reached in the last section. that the closed period is determined chiefly by oxygen want (carbon dioxide contributing to a small extent), whereas the open period is determined mainly by the time taken for carbon dioxide to diffuse away. A small amount of carbon dioxide in the outside air, by diminishing the gradient of partial pressures inside and outside the insect, will increase the time necessary for diffusion

This effect upon the open period is well seen when the flea is restored to air after exposure to higher concentrations of carbon dioxide, fig 5, B–E Instead of the spiracle closing within a few seconds, as it does after

nitrogen, fig. 4, F, it remains open for a length of time which increases with the concentration of carbon dioxide that has been used (*see* Table III). This delay is clearly due to the time taken for the carbon dioxide to diffuse out of the tissues and tracheal system until it falls to the threshold level; it may perhaps be regarded as an exaggeration of the process which normally determines the duration of the open period.

TABLE III

Gas mixture	Duration of persistent opening on return to air, at 20° C seconds
N_2 with 0.8% O_2	5–7
2% CO_2 in air	20–27
5% CO_2 in air	31–38
10% CO_2 in air	36–42
50% CO_2 in air	85–97

Conversely, in 2% carbon dioxide the spiracle opens within 20 seconds, but it does not become fully dilated until some 25 seconds later, whereas in nitrogen complete dilatation is developed almost at once.

We have seen that oxygen want, unless very extreme, leads to a rapid rhythm and not to prolonged opening of the spiracles, fig. 4. The prolonged opening which follows muscular movement, fig. 3, C, is therefore probably due to carbon dioxide production. The duration of this opening is much longer at lowered oxygen concentrations, fig. 4, D, E; presumably because the resting p_H of the blood is lowered (*see* pp. 407, 414) and therefore a given quantity of carbon dioxide will raise the acidity above the threshold level for a longer time.

And the persistent opening which occurs at the height of digestion (p. 400) is also due, probably, to carbon dioxide; for exposure of such insects to pure oxygen does not bring about any increased closure.

EFFECT OF LACTIC ACID ON THE SPIRACULAR MOVEMENTS

Since a slight excess of carbon dioxide will shorten the closed period, it is clearly capable of supplementing the effect of deficient oxygen in causing the spiracles to open. This suggests that both oxygen want and carbon dioxide excess act in virtue of the acidity they produce. If that is so, lactic acid introduced into the blood might be expected to have the same effect.

This has been tested by placing a minute drop of Ringer's solution, containing lactic acid, on a slide coated with paraffin wax, decapitating

the insect, and immersing in the solution the cut surface of the prothorax. If the flea is then left for some 20 minutes in a moist chamber, the acid fluid is carried throughout the body by the circulation of the blood.*

The experiments were made upon unfed fleas in which the spiracles seldom open except after movement. When 0·135 M lactic acid in Ringer's solution was used, Th. II and III remained permanently open, Abd. ii–vii usually remained closed, Abd. viii showed an extremely rapid rhythm, such as occurs in gas mixtures very poor in oxygen, fig. 6, C. By using rather higher concentrations of lactic acid (0·34 M), Abd. ii–viii may be caused to remain permanently open, and yet the movements of the abdominal contents (the heart, stomach, rectum, Malpighian tubes) and the struggling movements of the whole abdomen show that the nervous system is still functional. On the other hand, Ringer's

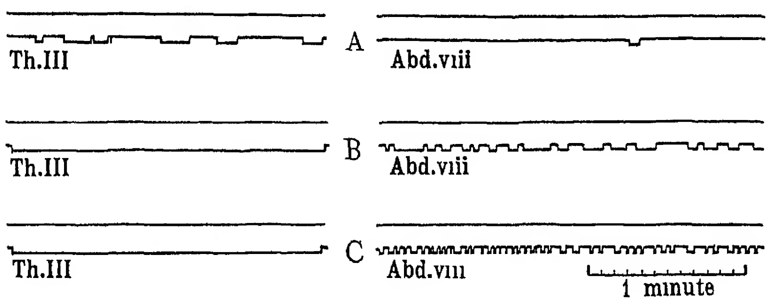


FIG. 6—Effect of lactic acid on movements of spiracles of unfed decapitated insects. A, exposed to plain Ringer's solution for 30 minutes; B, exposed to 0·135 M lactic acid in Ringer's solution for 30 minutes; C, exposed to the same for 45 minutes.

solution alone, or the lactic acid solutions neutralized with sodium hydroxide, have no effect upon the spiracles, fig. 6, A.

Now it has been shown by Davis and Slater (1928) and Bodine (1928) that in the complete absence of oxygen, insects gradually accumulate lactic acid, the p_H of the blood falling in the case of the grasshopper from 6·8 to 5·8. On readmission of oxygen the lactic acid, and perhaps other metabolites, are removed by oxidation, the oxygen consumption being increased above the normal until the accumulated "oxygen debt" has been paid off. These processes should be reflected in the behaviour of the spiracles.

This has been tested as follows. A little ring of paraffin wax, just large

* This can be proved by adding a little neutral red to the solution; the dye is taken up by the Malpighian tubes and by the fat body in the abdomen.

enough to enclose the flea, was drawn on a slide, and a corresponding ring was traced upon a coverslip supported at the corners with plasticene The flea was held within these superimposed rings by the pressure of the coverslip. Then by means of a fine pipette passing beneath the coverslip, the air was displaced so far as possible by nitrogen At the same time, alkaline pyrogallol was run under the coverslip The rings of paraffin, by abolishing capillarity, prevent the fluid from reaching the flea, which is thus enclosed in a small bubble surrounded by pyrogallol

The remaining traces of oxygen are soon removed by the tissues, on the one hand, and the pyrogallol on the other The spiracles remain permanently open, movements of the somatic muscles soon cease; and after about 10 minutes all the viscera come to rest, and the insect appears as though dead

In a typical experiment the flea was left in this state for 20 minutes. At the end of that time all the spiracles were still permanently open. The pyrogallol was then drawn off and air admitted. Within 30 seconds the degree of opening of all the spiracles was increased to the fullest extent, due perhaps to the additional stimulus of carbon dioxide, perhaps to the admission of oxygen reviving the nerve centres In $3\frac{1}{2}$ minutes, Th. II and Abd ii–vii began to close occasionally, Abd. viii was still fully open In 6 minutes Th. II showed a rapid rhythm in the absence of any leg movements; Abd. viii began to close momentarily from time to time In 10 minutes Th II showed a slower rhythm; Abd. vii was closed most of the time; Abd. viii showed a rapid rhythm; peristaltic movements of the stomach were just beginning In 15 minutes Abd. ii–vii were permanently closed; Th. II was closed nearly all the time, and Abd. viii showed a much slower rhythm In 20 minutes struggling movements were beginning; and the spiracular movements were not much different from the normal.

This experiment demonstrates that under conditions where it is known that lactic acid is accumulating in the absence of carbon dioxide (which will be absorbed by the alkaline pyrogallol) the spiracles remain persistently open The rapid rhythm which appears during recovery resembles the effect of oxygen want, fig 4, or of lactic acid introduced into the blood, fig 6, rather than the effect of carbon dioxide

Effect of Temperature on the Spiracular Movements

The effect of temperature has been studied by holding the flea beneath a coverslip upon a thin slide and examining this on a Reichert stage, the temperature of which could be regulated by passing through it iced brine

or warm water In the records below, the temperature quoted in each case is that of the stage The flea itself will certainly be a degree or two cooler at the warm end of the scale and somewhat warmer at the cold end

Fig. 7 shows a series of observations on a flea which made no struggling movements throughout the experiment and consequently gave very regular records The rhythmical movements are much accelerated as the temperature rises, both closed and open periods being shortened. Now

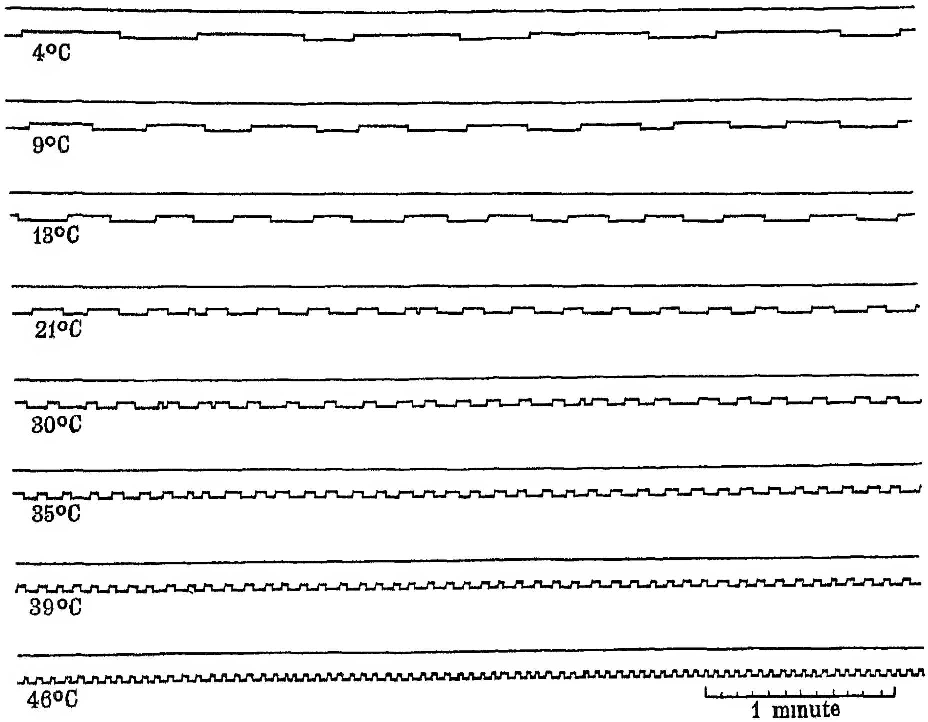


Fig. 7—Effect of temperature on movements of Abd viii The temperature given is that of the stage Note absence of struggling (upper line)

when the temperature falls below about 15° C *Xenopsylla* becomes exceedingly inert, as though paralysed, and the movements of the internal organs likewise diminish rather abruptly If, therefore, the duration of the *closed* period is determined by the rate of onset of oxygen want, we should expect this period to increase rapidly at low temperatures where the metabolism falls off abruptly Whereas, if the duration of the *open* period is determined by the time taken for carbon dioxide to diffuse away, since this in turn will be determined by such factors as the rate of diffusion (which bears a linear relationship to temperature), and the

solubility of carbon dioxide (the curve of which is inflected upwards only very gradually at low temperatures), we should not expect this open period to show a rapid increase as the temperature falls towards zero

In fig 8 the mean duration of the closed and open periods respectively is plotted against the temperature, and it can be seen that below 20° C the closed period increases very rapidly as the temperature falls, whereas the curve for the open period is little more than linear These results bear out the view that has been put forward They are not affected by the fact that the temperature of the flea will differ slightly from that of the stage

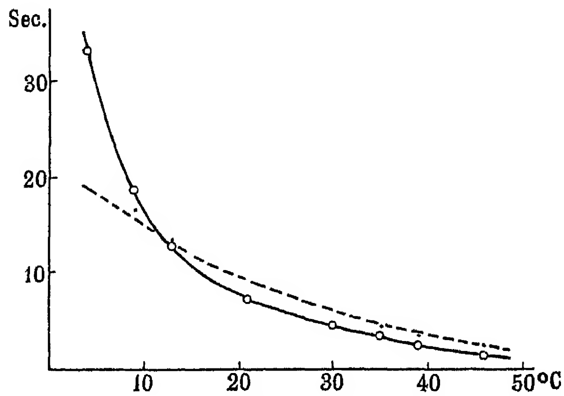


Fig 8—Mean duration of closed period —o—o and open period — — — in seconds plotted against the temperature of the stage

The Localization of the Respiratory Centres

Fig 9 shows the anatomy of the central nervous system of the flea as seen in longitudinal section Behind the brain there are three thoracic and seven (in the female) or eight (in the male) discrete abdominal ganglia. The last abdominal, being made up of several fused ganglia, is larger than those which precede it This hindmost ganglion lies less than half-way along the abdomen. Attempts to demonstrate the nerve supply to the spiracles by means of injections of methylene blue and rongalite white were unsuccessful, but from what is known of other insects it is reasonably certain that the spiracles will receive nerves from the ganglia of the segments to which they belong

For the cockroach, Hazelhoff (1926, 1927) has brought forward experimental evidence to prove that the regulation of the spiracles by carbon dioxide is a reflex response, the receptors of which are located in the spiracles themselves In the flea, no sense organs of any kind can be

found in the spiracles examined in whole mounts and in serial sections It seems more likely that in this insect the chemical changes to which the spiracles respond stimulate the nerve centres directly

With the object of locating these centres, various parts of the central nervous system have been removed After operating on the flea it is essential to seal the wound at once, or the tissues dry up very rapidly This can be done by preparing a wax of very low melting-point,* laying a minute fragment over the cut surface, and then bringing a warmed needle just close enough to melt it

After decapitation, fig 9, A, the responses to nitrogen and carbon dioxide are unchanged If cut through behind the metathorax so that

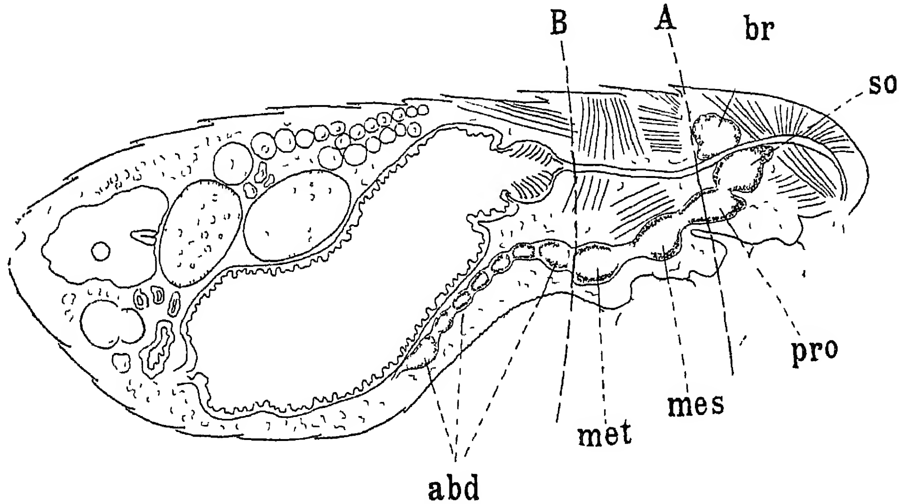


FIG 9—Longitudinal section of *Xenopsylla* to show anatomy of central nervous system. A, B, position of experimental sections, *abd*, adbominal ganglia, *br*, brain (supra-oesophageal ganglion), *mes*, mesothoracic ganglion, *met*, metathoracic ganglion, *pro*, prothoracic ganglion, *so*, suboesophageal ganglion

only the abdomen remains, fig 9, B, the same responses are given but the reaction is abnormally slow Thus, in the normal flea the spiracles will open after 5–10 seconds in nitrogen, and close in 5–7 seconds after returning to air; whereas in the isolated abdomen under the same conditions they may take from 23–30 seconds to open and from 13–17 seconds to close Moreover, after the thorax is removed, there is often a period (of nervous shock) during which no response can be obtained; or during which Abd viii can be caused to open but not Abd. ii–vii Clearly the

* A mixture of equal parts of vaseline and the " Sira wax " of British Drug Houses has been used

respiratory centres controlling the abdominal spiracles are located in the abdominal ganglia, but some influence is exerted upon them by the anterior segments of the central nervous system

If the whole of the central nervous system is removed by cutting half-way through the mesothorax without severing the nerve cord, and then drawing out the chain of abdominal ganglia entire, the normal rhythmic movements in the viiith abdominal spiracle cease The spiracle remains either closed or, more commonly, very narrowly open the whole time Immediately after this operation Abd viii will not respond to nitrogen or carbon dioxide. But after an hour or two a very slow reaction returns In one experiment, 5 hours after removal of the central nervous system, Abd viii would open widely after exposure to nitrogen for 1½ minutes, and on readmitting air it would revert to its almost closed position in 30–45 seconds And after exposure to 5% carbon dioxide for 2½ minutes it would open appreciably and return to normal again 2 minutes after it was restored to air No effect on Abd. ii–vii was obtained after removal of the abdominal ganglia

The explanation of these results is obscure Perhaps the spiracles receive a secondary nerve supply from the visceral nervous system; perhaps there are ganglion cells between the abdominal ganglia and the spiracles (as described by Landois and Thelen (1867) in the lepidopterous larva, *Cossus*) which are capable of stimulation There are, indeed, small cells like nerve cells, with nerve fibres among them close to the spiracular muscles, fig 2, B. But the nature of these cells is uncertain. It is clear, however, that there is a progressive reduction in the sensitivity of the respiratory control as more and more of the central nervous system is removed; and the main centres for controlling the abdominal spiracles appear to lie in the abdominal ganglia

Changes in the Tracheoles at Different Oxygen Pressures

It is generally believed that the greater part of the gaseous exchanges of insects, particularly the absorption of oxygen, takes place through the tracheoles Now in two previous papers (Wigglesworth, 1930, 1931) it has been shown that the terminal parts of many of the tracheoles of insects normally contain water, which is drawn up them by capillarity until the diameter of the vessels is such that this force exactly balances the osmotic pressure of the blood acting through their semipermeable walls During muscular exertion, particularly if the insect is asphyxiated by lack of oxygen, the production of metabolites raises the osmotic

pressure, water is then drawn down into the finer parts of the tubes until capillarity and osmotic pressure are in equilibrium once more

Conversely, the movement of water up and down the tracheoles affords an index of the changes in osmotic pressure that are taking place, and so of the efficiency with which the metabolites are being reconstituted or otherwise removed In other words, the changes in the tracheoles provide a criterion of an oxygen debt It is interesting, therefore, to consider these changes in connection with the present study, since they afford some indication of how far the regulation of the spiracular movements compensates for changes in the composition of the outside air in maintaining metabolic equilibrium in the tissues.

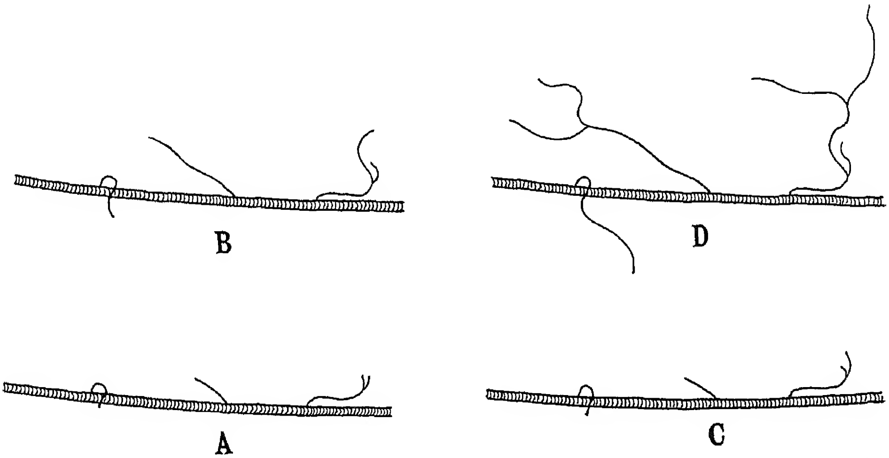


FIG. 10—A, extent of gas in tracheoles of flea at rest in air at 17 5° C, B, the same after struggling, C, the same in insect at rest at 35° C, D, the same 5 seconds after commencement of struggling (The distal parts of tracheoles, containing fluid, are invisible)

Fig. 10, A, shows the extent of air in a typical tracheole below the abdominal wall at 17·5° C. During muscular activity the air may extend a little further downward—as in fig 10, B, and then after a few minutes' rest it will return to about the previous level On raising the temperature to 35° C the movements of air which follow muscular effort are much more extreme and much more rapid Indeed, in many of the tracheoles, the meniscus is moving up or down almost the whole time The rapidity of these movements is illustrated in fig 10, C, D Five seconds after the onset of struggling, the air extended from position C to position D Struggling ceased almost at once, and 20 seconds later the fluid had risen again to position C But there is no change in the extent of the air when the flea is at rest. There is, therefore, no permanent accumulation of

metabolites the increased rate of diffusion and of chemical action at the higher temperature, coupled with the increased frequency of the spiracular movements (p 409), compensate for any increase in metabolism

On the other hand, when the oxygen in the air is reduced to say 10%, the equilibrium position in the tracheoles, when the flea is at rest, is definitely changed, the air extends more deeply into the tissues, fig. 11 In a 5% mixture of oxygen the air extends still farther, fig 11; and in nitrogen containing 0 8% of oxygen the air reaches such fine tubes that its limit cannot be seen with the microscope, fig 11

Clearly 5% and 10% of oxygen is insufficient, despite the more frequent opening of the spiracles (p 402), to prevent some accumulation of meta-

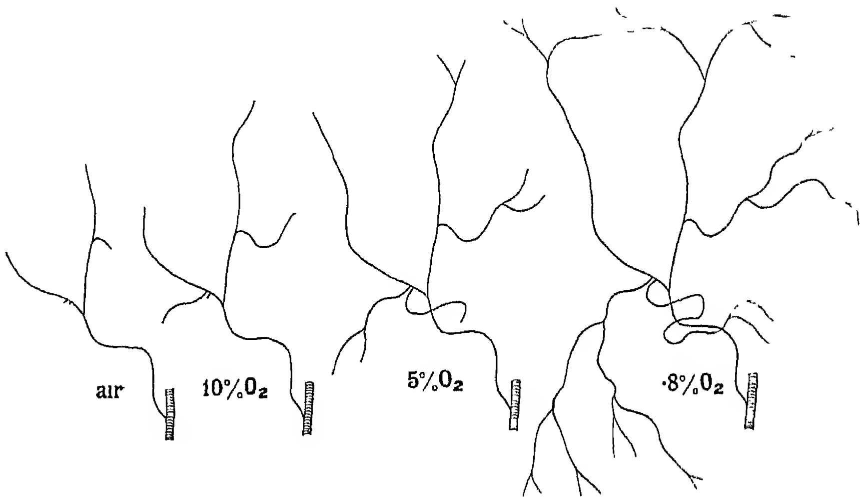


FIG 11—Extent of gas in tracheoles of flea at rest in different concentrations of oxygen in nitrogen

bolites But this accumulation is evidently arrested at a point which depends upon the partial pressure of oxygen in the atmosphere. Equilibrium between oxygen supply and oxygen consumption is then restored, a constant level of oxygen debt remaining.

The physiological processes concerned in this adaptation to reduced oxygen will be discussed later For the moment we may note merely that such reduction causes an accumulation of metabolites above what obtains in air That raises the question whether even in air a certain accumulation exists which can be removed in oxygen. Fig 12 shows that this must be so; for on going from air to oxygen the equilibrium point to which air extends in the tracheoles retreats appreciably towards the wider part of the tubes.

Discussion

The necessity for controlling the gaseous exchanges in such an insect as the flea results from the need for retaining water (Hazelhoff, 1926, 1927). For the greater part of the evaporation from insects takes place through the spiracles (Koidsumi, 1934; Mellanby, 1934), and those conditions which have here been shown to cause the spiracles to remain permanently open have been found by Mellanby to cause a great increase in the rate of loss of water The object of the present work was to define the mechanisms by which the spiracles are controlled

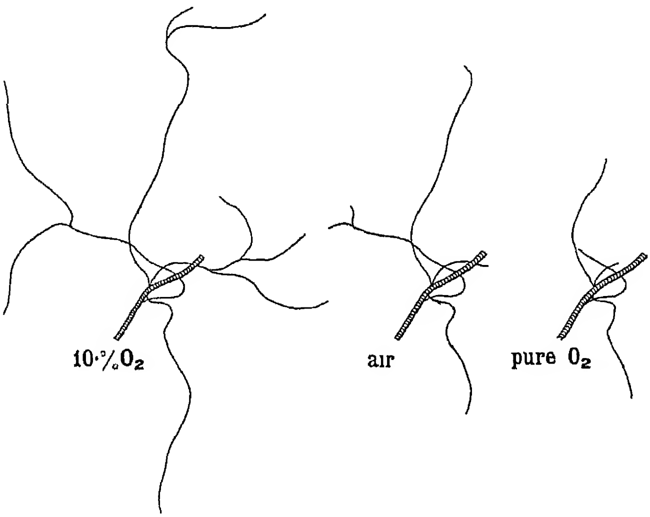


FIG 12—Effect of oxygen on extent of gas in tracheoles of flea at rest

In an insect with the spiracles permanently open, the tension of oxygen in the tissue cells will be determined by the fall in partial pressure which is necessary to provide in a given time for a given volume of oxygen (i) to diffuse from spiracle to tracheole (Krogh, 1920), and (ii) to invade the body fluids and diffuse into the tissues The tension of carbon dioxide in the cells will be determined by the rapidity of the same processes in the reverse direction Now the diffusion of carbon dioxide in air, being inversely proportional to the square root of the density, is not markedly different from that of oxygen, but its rate of diffusion through animal tissues is 35 times greater (Krogh, 1919). Hence the partial pressure difference necessary to ensure the transport of a given volume of oxygen will be very much greater than that needed for the transport of the same volume of carbon dioxide. And thus at a given moment the deficit in

oxygen tension in the cells, as compared with the atmosphere, will tend to be much greater than the carbon dioxide excess

Similarly, in an insect with controlled spiracles, it will be largely a question of anatomy whether the respiratory centres are stimulated by carbon dioxide accumulating up to the threshold level, or whether they are stimulated by oxygen want before that threshold has been reached

It is to be expected, however, that in most insects, with a tracheal system of very small capacity as compared with the body volume, oxygen want will provide a more sensitive index of increased oxygen consumption than the production of carbon dioxide For if, in fig 13, the tissues at A become active, oxygen will flow to this point from the entire tracheal system, and the centre at B is likely to suffer from oxygen want, and bring

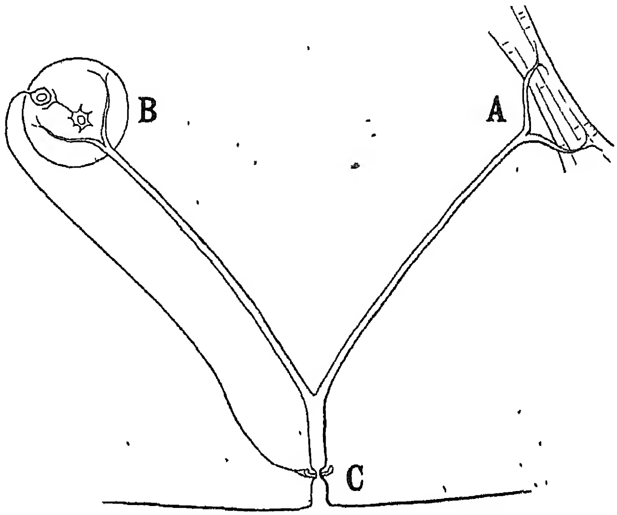


FIG 13—Diagram showing regulation of spiracles in an insect with a tracheal system of small capacity For explanation *see* text

about opening of the spiracle at C, before carbon dioxide in sufficient quantity has had time to reach B either by the blood or the tracheal system.*

That is found to happen in the flea. the spiracles are usually caused to open by oxygen want, though the removal of carbon dioxide is probably a main factor in determining how long they remain open.

The flea shows several adaptations to diminished concentrations of oxygen. (1) Oxygen want is more quickly felt when the spiracles are

* The conditions are very different from those in Vertebrates, where there is an efficient circulatory system, a carrier always more or less saturated with oxygen in the blood, and an oxygen store in the lungs

closed Hence the centres are stimulated sooner and the spiracles open more frequently (ii) There is an incomplete oxidation of metabolites, which increases the osmotic pressure of the blood, and this, by extracting water from the tracheoles, increases the respiratory surface (iii) This same incomplete oxidation probably lowers the p_{H} towards the threshold level of stimulation of the centres, and in this way contributes to the more frequent opening of the spiracles (iv) The accumulation of metabolites indicates that the oxygen tension in the tissues is reduced By increasing the difference in partial pressures, this will increase the rapidity of diffusion from the spiracle All these changes together serve to compensate for deficient oxygen in the air.

The nervous system, so far as it controls the respiration, is highly integrated The main centres controlling the abdominal spiracles certainly lie in the abdominal ganglia, but the sensitivity of these centres is influenced to some extent by the ganglia of the thorax The co-ordination of the system is seen in the predominance of the third thoracic, Th III, and the last abdominal spiracle, Abd VIII These alone are functional as a rule; and only when the stimulus is greatly increased does it affect Abd II–VII (p 401) Similarly, after anaerobiosis Abd II–VII recover their normal state sooner than Abd. VIII (p. 408) Presumably the centres for these spiracles have a higher threshold, or are stimulated only by a strong stimulus spreading from the main respiratory centres No peripheral receptors, which might detect carbon dioxide in the spiracles, such as Hazelhoff (1926, 1927) believes to exist in the cockroach, could be discovered, but the slow reactions of the last abdominal spiracle in insects deprived of the central nervous system (p 412) recall the responses obtained by Hazelhoff when isolated spiracles were exposed to high concentrations of carbon dioxide The possibility exists that the sensitivity of this peripheral mechanism may be very much greater when the nervous system is whole

From the standpoint of comparative physiology, it is perhaps worth while drawing attention to the fundamental similarity between the mode of action of oxygen want and carbon dioxide excess in the flea and in mammals. There is the same abruptness of response in the flea when regulation is due solely to oxygen want, as is seen in man at low oxygen pressures; and under normal circumstances the solubility of carbon dioxide in the tissue fluids results in that same slowing up of the responses which is characteristic of this gas as a respiratory stimulant in mammals (Haldane and Priestley, 1935).

SUMMARY

The anatomy of the tracheal system of the flea and the closing mechanism of the spiracles are described.

In the absence of muscular movement the third thoracic, Th III, and last abdominal spiracles, Abd viii, usually show a rhythmical opening and closing. The other spiracles are generally closed

During muscular exertion, Th I and II open, and the opening of Th III and Abd viii is prolonged Such opening occurs after activity has begun, sometimes after it has ceased

At the height of digestion all the spiracles of the abdomen may show rhythmical movements or remain permanently open.

Diminished concentrations of oxygen increase the frequency of opening and closing, the closed period being shortened more than the open period. Oxygen must be less than 1% before the spiracles remain permanently open. Increased concentrations prolong both open and closed periods

Low concentrations of carbon dioxide shorten the closed period slightly and increase the open period considerably Concentrations of about 2%, the exact figure depending on the intensity of metabolism, cause the spiracles to remain permanently open

Lactic acid introduced into the blood causes a rapid rhythm-like oxygen want, or permanent opening of the spiracles.

High temperature quickens the spiracular rhythm At low temperatures the closed period is lengthened more than the open period

It is concluded that the spiracles are caused to open chiefly by oxygen want (carbon dioxide contributing to a small extent) but that the duration of the open period is determined mainly by the time taken for carbon dioxide to diffuse away

The respiratory centres of the abdominal spiracles are probably localized in the abdominal ganglia, but the integrity of the nervous system seems to be necessary for the responses to be entirely normal Very feeble responses to abnormal gas mixtures remain even after the central nervous system has been completely removed

When the oxygen concentration is reduced, there is some accumulation of metabolites in the blood The resulting rise in osmotic pressure, by extracting water from the ends of the tracheoles, leads to an increase in the respiratory surface This and other adaptations to reduced oxygen pressure are discussed

REFERENCES

Bodine, J H. (1928) 'Biol Bull,' vol 55, p. 395

Davis, J G, and Slater, W K (1928) 'Biochem J,' vol 22, p 331

Fraenkel, G (1932) 'Z vergl Physiol,' vol 16, p 371.
Haldane, J S, and Priestley, J G (1935) "Respiration," Oxford, 1935
Hazelhoff, E H (1926) "Regeling der ademhaling bij insecten en spinnen." Thesis, Utrecht, 1926
—— (1927) 'Z vergl Physiol,' vol 5, p 179
Koidsumi, K (1934) 'Mem Fac Sci Agric. Taihoku Imp Univ,' vol 12, p 1.
Krancher, O (1881) 'Z wiss Zool,' vol 35, p 505
Krogh, A (1919) 'J Physiol,' vol 52, p 391.
—— (1920) 'Arch ges Physiol,' vol 179, p 95
Landois, H, and Thelen, W (1867) 'Z wiss Zool,' vol 17, p. 185
McGovran, E R (1931) 'Ann Ent Soc Amer,' vol 24, p 751
Mellanby, K (1934) 'Proc Roy Soc,' B, vol 116, p 139
Wagner, J (1889) 'Horae Soc Entom Ross,' vol 23, p 199
Wigglesworth, V B (1930) 'Proc Roy Soc,' B, vol 106, p 229
—— (1931) 'Proc Roy Soc,' B, vol 109, p 354

551 464 546 72

Iron in the Sea and in Marine Plankton

By L. H N Cooper

(*Communicated by W. R G Atkins, F R S—Received May* 29, 1935)

I

Gran (1931, 1933) believes that the greater production of phytoplankton in coastal as compared with oceanic waters may be due to organic compounds of iron washed out from the land and in favour of this he has found a fair amount of experimental evidence Agreeing well with Gran's thesis was Harvey's work on *Nitzschia closterium* (1933). The rate of growth of this diatom was much accelerated by enriching the cultures with small amounts of ferric ammonium citrate, ferric alum, or ferrous sulphate. A small piece of steel placed in the culture was also able to speed up growth Both Gran and Harvey have found that soil extracts improve the growth of diatoms Gran (1933) made a number of experiments with Waksman's synthetic "ferri-ligno-proteid" which has the same biological and chemical reactions as have the essential components of humus and found it to have a marked effect on the growth of certain diatoms

Studies on the growth of *Chlorella* sp. by Hopkins (1930) in cultures containing added iron salts and a citrate in many different proportions showed that iron is effective physiologically only in the ionic form.

Existing data on the iron content of the sea are scanty and the earlier records are untrustworthy. Very much lower values were obtained from two samples taken from the English Channel, about 0·1 mg and 0·2 mg of Fe per litre respectively were estimated (Orton, 1923). There is reason to believe that these values are too high. Harvey (1925) also in the English Channel detected between 3 and 6 mg per cubic metre, and off the coast of Norway in 63° N Braarud and Klem (1931) obtained values ranging from 4 to 23 mg in unfiltered water and from 4 to 9 mg in water filtered through glass Gooch crucibles (IG3 and IG4). In the South Atlantic Wattenburg (1927) found around 60 mg, but considers this likely to be high. At the surface at Friday Harbour, Puget Sound, there is a seasonal variation between a maximum of 60 mg per cubic metre in the spring and a minimum of 32 mg in July, the average for the whole year being 42 mg (Thompson, Bremner, and Jamieson, 1932). These results the authors believe to be low. In the very turbulent waters of the straits and archipelagos of their area they found about 40 mg in the surface increasing with depth, in one case to 280 mg per cubic metre at a depth of 200 metres. For a variety of reasons this area is extraordinarily rich in nutrient salts and conditions are seldom comparable with those in the English Channel. Little has been done on the distribution of iron in marine plankton. Sjostedt (1921) gave a list of 114 marine algae and phanerogams which contain iron mostly as iron oxide or hydroxide. Czapek (1920) gave a list of the iron contents of a number of marine plants. A number of analyses of marine organisms by Brandt and Raben (1920) will be referred to later.

Although the demand of the diatoms for iron is small, the supplies available in the water are also small.

II—Iron in Sea Water

Determination of Various Fractions—Until recently the only method at all suitable for determining the very small amounts of iron present in sea water was by colorimetric comparison of ferric ferrithiocyanate. This substance is of variable composition, its formation is suppressed by fluorides and its shade is very dependent on the concentration of other salts present. Thompson, Bremner, and Jamieson (1932) have carefully examined the method and find it satisfactory when closely prescribed conditions are adhered to. Their method requires the evaporation of the water acidified with sulphuric acid until fuming occurs. The oxidation of organic matter is then completed by potassium permanganate.

Thus the method is not readily applicable to the determination of the several forms in which iron may exist in sea water

Hill (1930) has suggested the use of 2 2'-dipyridyl for the determination of iron in biological material and Muller (1933) has used this reagent for determining iron in lake waters Dipyridyl forms a deep red co-ordination compound with iron which is reported to be very stable in the p_H range 3 5–8 5 (Hill, 1930) and with it conditions may be readily adapted for the determination in sea water of various iron fractions As little as 2 mg iron per cubic metre can be determined with a fair degree of certainty and no evaporation is needed

An allied substance, 2 2'. 2''-tripyridyl, has been prepared by Morgan and Burstall (1932) as a by-product in the large-scale preparation of 2 2'-dipyridyl by the dehydrogenation of pyridine, and this gives a similar violet co-ordination compound with ferrous ion For the determination of fair amounts of iron, tripyridyl has no advantage over dipyridyl, but when it is desired to determine the smallest amount possible, tripyridyl has two advantages Firstly, in a clear solution it is about twice as sensitive, bringing the lower limit down to about 1 mg Fe per cubic metre Secondly, at very high dilutions the violet colour of ferrous tripyridyl still appears violet whereas the red dipyridyl appears somewhat brownish and is liable to be obscured if the water is at all brown with plankton or detritus In such a case accurate determinations on less than 5 mg can be made only with tripyridyl

Thanks are due to Dr G T. Morgan, Director of the Chemical Research Laboratory, Teddington, for generous gifts of 2 : 2'-dipyridyl and 2 · 2'. 2''-tripyridyl

Reagents and Apparatus—1% 2 2'-dipyridyl (or 2 2' 2''-tripyridyl) in 0 2 N hydrochloric acid

4 N hydrochloric acid

4 N ammonium acetate preserved with a few drops of toluene.

10% sodium sulphite (freshly prepared)

Bromine water (freshly prepared) All the bromine must be in solution but otherwise the exact strength is immaterial Since bromine water has considerable solvent action on the iron in glass the reagent bottle was stored full of bromine water and washed out only when required for a fresh preparation

All the above reagents should be as free as possible from iron B D H., A R , and Analar reagents have proved very satisfactory

Glass beads which have been boiled with acidified bromine water. These after use are well washed and used again

Stock Standard Solution—Ferrous ammonium sulphate (0 7023 gm) dissolved in 1 litre of water to which has been added 5 cc of 4N hydrochloric acid This solution contains 100 mg of iron per litre and is diluted when required.

Monax glass-ware was used throughout New flasks were first left full of cleaning mixture for a few days, then filled with bromine water, covered with an inverted glass beaker and warmed on the steam bath for 24 hours

Determination of Ferrous Iron—The sample of sea water, 100 cc, was treated with 2 drops of 4N hydrochloric acid and 1 cc of 1% tripyridyl solution and left for 24 hours A similar solution was made with distilled water and many of the samples were found to be identical with this, showing that ferrous ion was less than 1 mg per cubic metre.

Determination of " Reducible Iron "—The sample, 100 cc, was treated with 4 drops of 4N acid and 1 cc of 10% sodium sulphite. After 20 minutes 1 cc of tripyridyl solution and 4 drops of 4N ammonium acetate were added and the sample left for 24 hours This treatment determines not only ferrous and ferric ions but also certain forms of insoluble iron

Determination of Total Iron—The sample, 100 cc, from a well-shaken bottle was treated with 4 drops of 4N hydrochloric acid and 1 cc of bromine water and warmed on the steam bath for half an hour A glass bead was added, together with 5 cc of distilled water to compensate for the subsequent loss in boiling off the excess of bromine The sample was cooled and treated with 1 cc of 10% sodium sulphite followed after 20 minutes by 1 cc of 1% tripyridyl solution and 4 drops of 4N ammonium acetate It was then set aside for 24 hours.

In order to determine the bromine blank correction a sample of distilled water was treated in the same way but using only 2 drops of hydrochloric acid With a freshly prepared sample of bromine water this was usually negligible.

Determination of Iron in Filtered Water—In the earlier work the water was filtered through a No. 40 or 42 Whatman filter paper which may let through bacteria, small plants, and other particulate matter so that the results meant little Centrifuging for 15 minutes at 1800 r p.m has not been found to be an efficient means of removing very fine particles. Much more concordant results were obtained by filtration through a 9-cm membrane filter (from the Membranfilter Gesellschaft, Göttingen) having a filtration constant of 20 seconds. The subsequent treatment was exactly as for total iron.

Since the iron content of membrane filtered water was found to be very low, tests were made to discover whether the filters adsorbed iron from solution. As the subsequent discussion will show the amount of ferric ion that can exist in sea water is far less than could be detected by tripyridyl. If, therefore, a solution of ferric chloride were added to sea water ferric hydroxide would be at once precipitated. When known amounts of "ferric and ammonium citrate" (in which the iron is in the anion) were added to filtered sea water about 75% of the added iron was found after filtration through a membrane filter, Table I. However, the stock solution of the citrate on standing for several weeks was found to have deposited a considerable quantity of a brown precipitate, suggesting that not all of the citrate had been in true solution and accounting for the 25% loss on filtration.

TABLE I—EFFECT OF FILTERING SOLUTIONS OF IRON IN SEA WATER THROUGH MEMBRANE FILTERS

Ferrous sulphate			Ferric and ammonium citrate		
Added	Recovered		Added	Recovered	
mg/m³ Fe	mg/m³ Fe	%	mg/m³ Fe	mg/m³ Fe	%
0	0	—	0	0	—
5	5	100	10	10	100
10	11	(100)	19	14	73
20	15	75	38	27	71
40	28	70	77	57	74

A similar experiment was carried out with added ferrous sulphate. In order to minimize the effect of hydrolysis and oxidation filtration was carried out immediately after the addition. It will be seen from Table I that when only 5 or 10 mg of ferrous iron was present complete recovery was effected in the filtrate. With larger amounts there was some loss on filtration. This loss was probably due to hydrolysis to the sparingly soluble basis salt, discussed on p. 434, which was retained by the filter. The experiments were not so conclusive as could be wished but they indicate that small amounts of ferrous iron can be filtered without loss, that larger amounts cannot be filtered without some loss since such concentrations cannot exist in solution in sea water, and that iron in the complex citrate can be filtered when in true solution.

Although when sea water is filtered through a membrane filter some loss of iron in true solution due to adsorption may possibly occur, this loss is not sufficient to destroy the value of the results.

Methods of Comparison with Standards—At such high dilutions the ferrous tripyridyl takes a considerable time to form, and standards and samples were best set aside for at least 24 hours for the full colour to develop Since Beer's law does not apply rigidly at the highest dilutions, comparisons of samples containing less than 10–15 mg iron per cubic metre were made in the bell-topped tubes 35 cm high, which were used for the determination of ammonia (Cooper, 1933, p 719) The standards at close intervals were made by the procedure described for reducible iron

In work on samples richer in iron, lower standards were compared with higher in Hehner tubes, and calibration curves constructed from which the strength of unknown solutions could be read

Results are accurate to within $\pm$ 1 mg per cubic metre ($\pm$ 1 γ per litre) and there is no salt error

Interference by Fluoride—Fluoride interferes seriously with many methods for determining iron and it seemed possible that ferrifluoride might prove more difficult to reduce than ferric iron Feigl and Hamburg (1931) state that the reduction of ferric iron by sulphur dioxide in solutions containing fluoride is not possible However, the writer has found that this is true only in solutions containing more than 100 mg fluoride ion per litre. In solutions containing 10 mg fluoride ion per litre or less, ferric iron is reduced quantitatively by the procedure described above, although the rate of colour development with the pyridyl reagents is markedly affected Since fluoride in sea water amounts to only 1 5 mg per litre, its influence may be neglected, providing that 18 hours or more elapse before colour comparison is made

Collecting Methods—The stations worked are on a course S. 37° W. from Plymouth The distances from the Laboratory in nautical miles are :—L 4, 8 , L 5 (Eddystone), 12, L 6, 17 , E 1, 22, E 2 (mid-Channel), 56. Surface samples were collected in a wooden bucket and the remainder in a Nansen-Pettersson insulating water-bottle which has no iron parts. The samples were stored in the dark green glass bottles which have been in use here for many years, and particular care was taken to avoid contamination from the galvanized iron stopper spring Although both storage bottles and Monax glassware probably contain iron silicates it was considered that contamination was likely only with new glass

Discussion of Sea Water Results

The total iron present in all forms was very erratic in its distribution during the winter months when the water is isothermal from top to

bottom and dissolved substances are usually very uniformly distributed due to vertical mixing Not only is the vertical distribution erratic but duplicate determinations often differ by much more than the experimental error

In January and February, 1934, samples were filtered through Whatman's No 40 filter paper On the first occasion the iron content of filtered water was less than the total but equal to the reducible fraction; on the second occasion iron was almost undetectable although some iron may conceivably have been lost by adsorption on the filter paper

On March 20 and November 14 the water was filtered through a 9-cm diameter membrane filter having a filtration constant of 20 seconds On these occasions also, iron in solution was practically undetectable, but the results are unlikely to be due to loss by adsorption on the filter, *cf.* p 423. Thus on these dates and probably on February 12 iron in solution was less than 1 mg per cubic metre

The difference between determinations of total iron in membrane-filtered and in unfiltered water gives a measure of the "particulate iron" and in this large variations were found An occasional high figure such as that at the surface at Station E1 on February 12, 150 mg per cubic metre, may have been due to contamination On the same date for the surface at 42° 42′ N, 4° 28′ W, high figures were found not only for total iron, 100 mg, but also for reducible and ferrous iron, 24 mg, whilst the iron content of filtered water was nil This sample was therefore exceptionally rich in particulate iron

On most occasions the reducible iron was uniformly distributed from top to bottom, but on March 20, when the whole of the iron was reducible, a large excess was found at the bottom due probably to dead plankton organisms and faeces sinking to the bottom during the spring outburst. Iron is known to be excreted by the zooplankton more rapidly than phosphorus (pp 427–429) and probably returns into circulation more quickly. Ferrous iron was often undetectable and when, as on November 14, 1934, it exceeded iron in solution a portion of this ferrous iron must have been present in an insoluble form from which free ferrous iron was liberated during the course of analysis

Thus iron in true solution in the water as ferrous and ferric salts seems to be very small, probably less, possibly much less than 2 mg per cubic metre Most of the iron is present in particulate form, the amount of which varies greatly Some of this is reducible by the acidified sulphite at p_H 3 5–4 and a small ferrous fraction may be set free in the presence of acid and tripyridyl alone at about p_H 3 Iron is probably present in both states of oxidation in the particles which may consist of colloidal

ferric hydroxide (Harvey, 1925, Braarud and Klem, 1931) and living and dead organic matter

On March 20, 1934, the water column had an average total iron content of 14 mg per cubic metre and of this 16% (2 3 mg) was present in the plankton caught in the quantitative plankton net On July 9 only 6% of the total iron was present in the form of plankton.

During the spring outburst of phytoplankton more iron than phosphorus is required by the plants (pp 427–429) so that a fall in iron content

TABLE II—AVERAGE IRON CONTENT OF WATER COLUMN, MG FE PER CUBIC METRE

Date	(1) Ferrous iron	(2) Reducible iron	(3) Iron in solution in filtered water	(4) Particulate iron, difference of (3) and (5)	(5) Total iron
Station L4—					
December 15, 1933	2 5	11	—	—	15
January 22, 1934	—	10	(8)*	—	23
February 12, 1934	—	7 5	(8 5)*	14	22
March 20, 1934	—	12	1†	9	10
July 9, 1934	5	3 5	—	—	9
November 14, 1934	—	7	—	—	—
Station E1—					
November 13, 1933	0	—	—	—	—
December 15, 1933	0 5	4	—	—	8
January 22, 1934	—	9	(9)*	12	21
February 12, 1934	0 5	7	(0)*	*ca* 25	*ca* 25
March 20, 1934	—	9	1†	8	9
July 9, 1934	$\not>$4	6·5	—	—	6
November 14, 1934	1	3 5	0†	4	4

* Analyses on water filtered through Whatman No 40 papers.
† Analyses on water filtered through a 20-seconds membrane filter

of the water would be expected. From the available data, Table II, no clear picture can be drawn since we cannot follow the changes in iron in solution in the way we are accustomed to follow the changes in dissolved phosphate, silicate, nitrate, and ammonia The assimilation of iron by phytoplankton and its transference through smaller to larger animals would be without effect on the total iron content of a mass of water inclusive of everything in it But in practice, whereas the phytoplankton and the smaller zooplankton organisms will be taken with a

sample of water and included in the analysis for total iron, the larger animals and the bottom dwelling species, feeding on the rain of food from above, will escape. The average total iron content of the water column as found by analysis should therefore decrease. Something of this sort did happen between February 12 and March 20, 1934, when the total iron content of the water column fell by more than half.

Since this paper was prepared further contributions have appeared by Thompson and Bremner (1935, *a* and *b*). They have improved their thiocyanate procedure and now report results for total iron and for soluble iron determined on water filtered through No. 42 Whatman filter papers. In the north-east Pacific and the Gulf of Alaska in summer for like depths their results are similar to those obtained here in January and February, 1934, when No. 40 Whatman filter papers were used. However, further work has shown (*see* above) that filter papers let through fine particles, possibly colloidal, which are held back by a membrane filter. Thus in comparing results for " soluble iron " it is important to take into account the method adopted for separating insoluble or particulate iron.

III—Iron in Plankton

Determination by 2 : 2′-*Dipyridyl*—The diluted digest from the work on phosphorus was used (Cooper, 1934). Ten cc of diluted digest was treated with 0·10 cc 4N hydrochloric acid and 0·20 cc freshly prepared 10% sodium sulphite. After 5 minutes 0·40 cc of a 1% solution of dipyridyl in 0·2N hydrochloric acid together with 0·20 cc 4N ammonium acetate were added. Four hours later the samples were compared in a Duboscq colorimeter with a series of standards containing between 0·1 and 3 mg iron per litre. For less accurate work the reagents may be measured in drops taking 0·1 cc as equal to two drops. The accuracy of the method was tested by comparing lower with higher standards, Table III. It will be seen that results average about 1% low so that Beer's law is obeyed with an accuracy sufficient for the present work.

Discussion of Plankton Results—Data extending from March 9 to October 31, 1934, are included in Table IV. The maximum amount of iron in plankton, 2·3 mg Fe in the catch from a cubic metre of water filtered, was found on March 20, 1934. During April, May, and late September the plankton contained about 1 mg per cubic metre filtered whereas during the summer only about 0·5 mg was present. With the approach of winter the iron content of the catch decreased rapidly. In the phytoplankton there was always several times as much iron as

TABLE III—COMPARISON OF STANDARDS

Standards compared mg Fe per litre A	B	Fe found in A when compared with B	% error
3·00	5·00	3·06	+2·0
1·50	3·00	1·48	−1·3
1·00	3·00	0·96	−4·0
1·00	1·50	0·97	−3·0
0·800	1·00	0·782	−2·2
0·667	1·00	0·623	−6·6
0·667	0·800	0·670	+0·5
0·500	0·667	0·495	−1·0
0·400	0·667	0·398	−0·5
0·400	0·500	0·405	+1·2
		Mean error	−1·3

TABLE IV—SEASONAL CHANGES IN IRON AND PHOSPHORUS CONTENT OF PLANKTON CATCHES TAKEN WITH THE QUANTITATIVE NET IN THE ENGLISH CHANNEL AT STATION L4 BETWEEN THE SURFACE AND 45 METRES

Date 1934	Content in plankton from a cubic metre of sea water		Ratio Fe/P
	mg Fe	mg P	
March 9	1·53	0·517	3·0
March 20	2·30	0·500	4·6
March 26	1·84	0·556	3·3
April 3	0·85	0·483	1·75
April 9	1·02	0·472	2·2
April 20	1·06	0·586	1·8
May 10	1·06	1·32	0·80
May 15	0·96	0·96	1·00
June 11	0·89	0·36	2·5
June 18	0·493	0·295	1·7
July 9	0·55	0·398	1·4
July 19	0·55	0·436	1·26
July 27	0·495	0·50	1·0
August 31	0·48	0·52	0·92
September 11	0·48	0·56	0·86
September 21	1·0	1·04	0·96
October 2	0·81	0·31	2·6
October 18	0·67	0·31	2·2
October 31	0·24	0·23	1·05
November 14	0·52	0·15	3·5

phosphorus, whereas in zooplankton the ratio was often less than unity, Table V The low and variable iron content of animals is also shown by the analyses of a few typical members of the zooplankton in Table VI. The animals were very kindly picked out alive by Mr F S. Russell from his townet hauls and transferred directly to the digestion flask. The iron content not unexpectedly varies from animal to animal but the relatively low iron content of large *Ctenophores* is noteworthy.

TABLE V

Date 1934	Composition of catch	Ratio Fe/P
March 20	20% faecal pellets, remainder diatoms	3 9
April 3	Almost entirely diatoms	4 2
May 24	Diatoms, mainly *Rhizosolenia*	4 4
April 20	Almost entirely animal	0 9
May 4	Almost entirely animal	0 56
May 10	Faecal pellets only	5 0

Brandt and Raben (1920) also made analyses on mixed plankton catches taken in the neighbourhood of Kiel Their analyses of iron were reported as Fe_2O_3 and included as Al_2O_3 any aluminium present. For nine catches, consisting mostly of diatoms, taken in August, 1902, or the autumn of 1908, the mean value of the ratio Fe/P was 1 41, somewhat less than at L4 in 1934 In another catch, mostly *Calanus*, the ratio was only 0 08, in one of *Anomalocera* it was 0 59, and in one of *Sagitta*, 0·24 So that at Kiel also the animals were poorer in iron relative to phosphorus than were the plants

At L4 the seasonal variations in the composition of the catch were reflected in the values of the ratio, Fe/P (wt/wt) When the catch is rich in plants the ratio is high; when it is poorer in plants the ratio falls As fig 1 shows, the parallel is very close in spring and autumn, much less so in the summer

The analysis of the faecal pellets separated from a catch by Mr. F S. Russell on May 10, 1934, showed that much more iron than phosphorus was voided by the animals Furthermore, Kostytsheff (Atwater 1892) found that the edible flesh of a number of marine and freshwater fish contained relatively little iron, the ratio Fe/P averaging 0 013 Similarly the analyses of McCance and Shipp (1933) on the cooked edible portions of fish show very little iron, the ratio averaging about 0 003. In neither case was " offal " or bone analysed, and the offal, in particular, is likely

to include much of the iron in the fish Even so it seems clear that the vertebrate fish, like zooplankton, need much less iron than phosphorus.

Thus, although the plants require several times as much iron as phosphorus, when the plants are eaten much of this iron will be returned to the water as faeces from which the iron may be liberated more or less

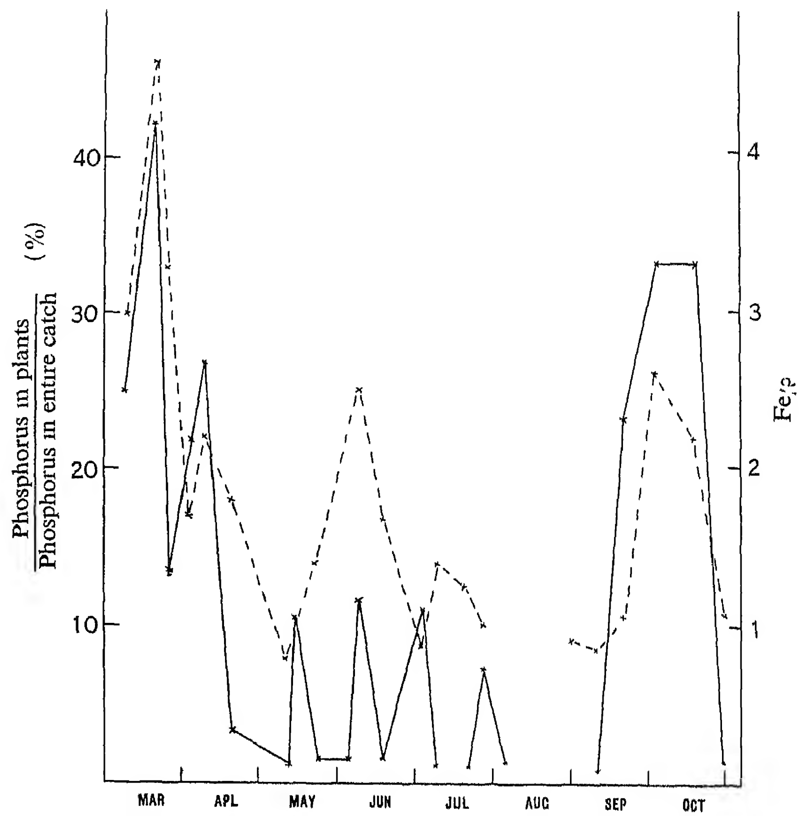


FIG. 1——— Proportion of plants to total plankton catch as measured by phosphorus content (Harvey *et alii*, (1935) - - - Ratio of iron to phosphorus in the catch

quickly to take part in another growth cycle. In the sea the iron and silicate cycles seem to have much in common In both cases the number of times which the same supply of silica or of iron can be used in one season turns upon the rate at which the stores in dead diatoms and in faecal pellets become available. Only so can the small amount of iron in the water support the diatom population

TABLE VI—PHOSPHORUS AND IRON CONTENT OF INDIVIDUAL ANIMALS

Date when caught 1934	No of animals in sample	Animal	Content of one animal γ Fe	γ P	Ratio Fe/P
May 28	20	♀ *Calanus*	0 64	3 2	0 20
May 24	6	*Sagitta setosa, ca* 15 mm long	6 8	5 3	1 28
May 18	4	*Phialidium*	5 0	4 8	1 04
		Ctenophores—			
May 18	4	*Pleurobrachia*	<2 5	30	<0 08
October 2	1	*Bolina infundibulum*	9 5	260	0 038
Nov 8	1	*Beroe cucumis* (*a*)	11 2	670	0 017
Nov 8	1	*Beroe cucumis* (*b*)	7 4	430	0 017
Nov 8	1	*Beroe cucumis* (*c*)	7 0	520	0 013

IV—THE CHEMISTRY OF THE IRON CYCLE IN THE SEA

Influence of Fluoride on the Iron System—According to Thompson and Taylor (1933), there is about 1 5 mg of fluorine per cubic metre of water and the amount present in the open sea is a linear function of chlorinity. The presence of fluoride must be taken into account when considering the iron cycle in the sea All fluorides are but slightly ionized and ferric fluoride probably less than most, so that, quite apart from complex formation, the concentration of ferric ion is likely to be greatly reduced by the formation of this salt Moreover, in the double fluorides there is no doubt that the iron or aluminium is part of the anion by the formation of which the concentration of free ferric or aluminium ion is very much reduced The formation of ferrifluoride is sufficiently complete to be used as the basis of volumetric methods for determining fluorine with thiocyanate (Greef, 1913) or with acetylacetone (Armstrong, 1933, and Wilcox, 1934) Although the ferrifluoride complex is stable enough for analytical purposes the latter authors point out that the equilibrium is not entirely in favour of the ferrifluoride as against the ferric acetyl acetone.

In considering the ferrifluoride system in sea water the presence of aluminium cannot be ignored According to a provisional figure obtained in Thompson's laboratory (Thompson and Robinson, 1932), there is about 0 07 milligram-atom of aluminium per kilogram of water of 19 0‰ chlorinity This figure may be high due to contamination from the glass It compares with 0 043 milligram-atom of fluorine. Since for the formation of sodium aluminofluoride six atoms of fluorine are

required for each atom of aluminium all the fluorine may well be in the form of aluminofluoride; the possibility of the formation of ferrifluoride therefore depends on the relative stability of the two complexes

In the presence of citrate Hopkins (1930) found the effective concentration of iron for the growth of *Chlorella* sp to be much reduced For the complex citrate he has established the formula $K_3[H_3(C_3H_4O)_3(CO_2)_9Fe_2]$, *i e*, 1 5 mols of citrate combine with each atom of iron When less citrate is present than is required by this formula the citrate appears to have reducing powers, for not only is free ferric ion present but ferrous ion as well By extrapolation he found that the minimum concentration of iron able to promote growth of *Chlorella* in his cultures was 1 47 × 10^{-6} gram-ion per litre, or 80 mg per cubic metre, and the iron requires to be in the ionic state This is several times the total amount of iron found in the English Channel and many times the quantity of iron in solution Marine diatoms must therefore be very well adapted to use such small amounts of iron as do occur

Limiting Concentrations of Free Iron Ions in Sea Water—The limiting concentration of a free ion in water will be controlled by the solubility of the least soluble compound which may be formed under the given conditions. For iron in sea water the hydroxides seem likely to be such compounds, and, although the direct determination of their solubilities would be a matter of some difficulty, data exist from which approximate calculations may be made.

(1) *Solubility of Ferrous Hydroxide*—The free energy of formation and the solubility of ferrous hydroxide are connected by the relation.

$$\Delta F^{\circ}_{Fe(OH)_2} = \Delta F^{\circ}_{Fe^{++}} + 2\Delta F^{\circ}_{OH'} + RT\,lnS,$$

where ΔF° is the free energy of formation of the substance shown by the subscript, R is the gas constant, T is the absolute temperature and lnS is the natural logarithm of the solubility product.

Existing data for these two quantities are given in Table VII. Of these data, those of Whitman, Russell, and Davis (1925) who determined the solubility in water of pure iron powder in deoxygenated solutions, and of Randall and Frandsen (1932) appear amongst the most reliable. The former authors found that the p_H of the saturated solution was 9 6, that the solubility product in pure water was 3 2 × 10^{-14}, and that the ferrous hydroxide in this solution was 30% ionized They also determined the effect on the solubility of the presence of certain other salts and found the results in agreement with those calculated by means of the ionic product. The correction for our purpose is, however, relatively negligible

TABLE VII—DATA FROM VARIOUS SOURCES ON THE SOLUBILITY PRODUCT AND FREE ENERGY OF FERROUS HYDROXIDE

Authority	Method of determination	Solubility product	$-\Delta F^\circ$ cals
Whitman, Russell and Davis (1925)	Direct solubility and p_H measurement	3.2×10^{-14}	113,600
Schrager (1929)	Polarographic	7×10^{-13}	111,900
*Muller (1908, 1917)	Calculated from Bodlander's formula (1898)§	1.64×10^{-14}	114,000
Krassa (1909)	From activities of ions in 20% KOH solution, [Fe^{++}] from e.m.f. measurements	8.7×10^{-14}	113,000
Lamb (1910)	Conductivity measurements	9.84×10^{-15}	114,300
Shipley and MacHaffie (1924)	p_H measurements	7.93×10^{-15}	114,500
Elder (1930)	p_H measurements	4.8×10^{-16}	116,100
†Britton (1925, *a*)	Electrometric titration	4.5×10^{-21}	123,100
‡Sweeney (1928)	Recalculated from Britton's results	1.6×10^{-19}	120,900
Randall and Frandsen (1932)	Free energy from e.m.f. measurements	1.7×10^{-15}	115,400
	Mean of $-\Delta F^\circ$ [excluding *, †, and ‡]	1.8×10^{-14}	114,100

§ Bodlander's formula is of very doubtful validity

Therefore in sea water of p_H 8·2, taking pK_w as 14, the concentration of hydroxyl ion will be $10^{-5·8}$ gram-ion per litre, so that

$$[Fe^{++}][OH']^2 = S = 3·2 \times 10^{-14}$$
$$[Fe^{++}] = 1·3 \times 10^{-2} \text{ gram-ion per litre}$$
$$= 0·7 \text{ gram per litre}$$

Consideration of all the data except those of Britton (1925) and Sweeney (1928) show that a figure of about 0·1 gram per litre is a most probable value for the maximum solubility of the ferrous-ion as controlled by hydroxyl, but using Britton's figure for the solubility product as recalculated by Sweeney (1928), $1·6 \times 10^{-19}$, we obtain only $6·4 \times 10^{-8}$ gram-ion per litre or 3·5 mg per cubic metre as the solubility of ferrous ion, a value enormously smaller than the above. Although this figure stands alone among the solubility data given, it is supported by other work of a different kind. Patten and Mains (1920) noted that when alkali was progressively added to ferrous sulphate precipitation occurred in the p_H range 5·5 to 6·0. Atkins (1923) in similar experiments found that the range of precipitation lay between p_H 5·1 and 7·0 approximately. Britton (1925, *a*), in the course of his electrometric measurements, found that precipitation began at p_H 5·49, although he considers that this represents the formation of precipitate at a point in the solution where the precipitation value of p_H had been temporarily exceeded and time not allowed for its re-solution. From his data he considers that p_H 5·77 truly represents the point of initial precipitation. There is thus very fair agreement between the results of the different investigators working along these lines. Britton's precipitate, like those of several earlier workers, was a basic salt, but he considered that his results were adequate for the calculation of the solubility product of the hydroxide. In view of the great discrepancy between his and other values of the solubility product, it appears probable that these basic salts are far less soluble than ferrous hydroxide and that it is not permissible to calculate the solubility product of the hydroxide from experiments involving the addition of alkali to a ferrous salt. If this is so, the solubility of ferrous ion in sea water will be controlled not by the solubility of the hydroxide but by that of an ill-defined basic salt in equilibrium with a solution of sea salts at a p_H of about 8. The figure, 3·5 mg per cubic metre, may therefore be taken as a measure—a very rough measure—of the limiting solubility of ferrous ion in sea water which shows, nevertheless, that the solubility of ferrous ion is of the same order as the actual quantity of iron found in sea water in the English Channel.

Ferrous ion set free in sea water will be oxidized more or less quickly to the still more sparingly soluble ferric hydroxide now to be discussed

(2) *Solubility of Ferric Hydroxide*—The solubility product of ferric hydroxide $S = [Fe^{+++}][OH']^3$ was found by Jellinek and Gordon (1924) to be $10^{-37.0}$ Britton (1925, *b*), making certain assumptions, calculated the value $10^{-37.7}$ from his electrometric data.

With a knowledge of the free energies of ferric hydroxide and ferric and hydroxyl ions it would be possible to calculate the solubility of the hydroxide Unfortunately, the free energy of the hydroxide is not known, neither is the entropy which would enable the free energy to be calculated Using what data there is, two different methods of calculation have given a probable maximum figure of about 10^{-42} for the solubility product of ferric hydroxide, a figure still lower than those of Jellinek and Gordon and of Britton. There must of necessity be some uncertainty as to the solubility of a substance of such colloidal habit, but nevertheless some interesting deductions may be drawn from the figures available.

TABLE VIII— LIMITING SOLUBILITY OF FERRIC ION IN SEA WATER

Source of data	Solubility product S	Sea water		Concentration of ferric ion		
		p_H	p_{OH}	gram-ions/l	mg/m³	ions/l
Jellinek and Gordon and Britton	$10^{-37.8}$	8	6	$10^{-19.8}$	10^{-12}	10^4
Cooper from thermodynamic data	10^{-42}	8	6	10^{-24}	$10^{-16.7}$	1

In Table VIII are shown the concentrations of iron in sea water as calculated from these values of the solubility product of ferric hydroxide. In the last column is given the number of individual ions present in a litre of sea water calculated with the aid of Avogadro's constant Although such a small concentration of ferric ion as 10,000 ions per litre or less may readily take part in instantaneous ionic equilibria, it is difficult to conceive how plants could assimilate sufficient iron for their needs from such a dilute medium If diatoms need dissolved iron, ferrifluorides remain as the only likely source, even though, as the present work suggests, the amount of iron so available is less than 2 mg per cubic metre of water. Since laboratory experiments have shown that the reduction of ferrifluoride at these concentrations is easy, this may well be the method used by the living plant. This hypothesis appears much more likely than

that the diatoms should be able to assimilate directly colloidal or suspended iron compounds of a type held back by a membrane filter.

These considerations are set out in diagrammatic form in fig. 2.

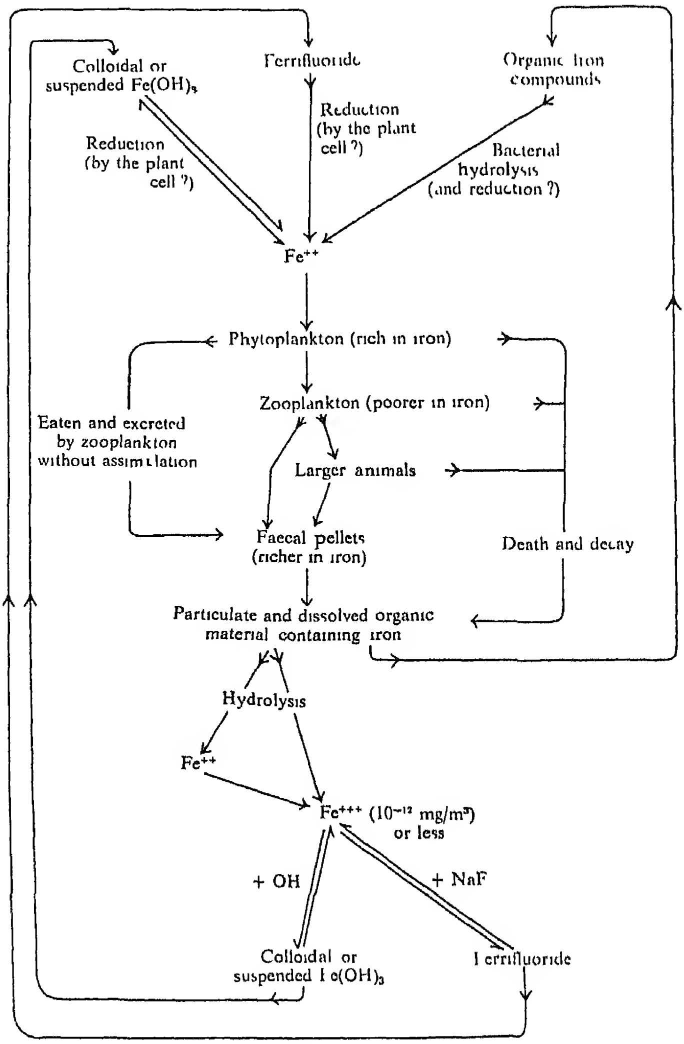


FIG. 2—Tentative diagram of the iron cycle in the sea

VI—SUMMARY

2:2′·2″-tripyridyl has been found to be a very sensitive reagent for the colorimetric determination of iron in sea water, the smallest amount determinable with certainty being 1 mg Fe per cubic metre. 2:2′-dipyridyl has also been used but is only about half as sensitive.

The amount of iron in true solution in sea water as ferric or ferrous salts is very small, probably less, possibly much less, than 2 mg per cubic metre. Most of the iron is present in particulate form, the amount of which varies considerably. On one occasion 16% of the total iron was present in plankton taken with the quantitative net.

The effect of the formation of complex fluorides on the iron system is discussed.

The solubility of ferrous ion in sea water is probably controlled, not by the solubility of the hydroxide but by that of a sparingly soluble basic salt. Ferric hydroxide is exceedingly sparingly soluble and the concentration of free ferric ion in sea water is unlikely to exceed 10^{-12} mg per cubic metre. Since the supply of iron ions in solution is so meagre, the availability for diatoms of ferrifluorides and colloidal iron compounds is discussed.

Diatoms require several times as much iron as phosphorus, whereas the iron requirements of zooplankton are smaller. At Station L4, between the surface and 45 metres depth, the plankton catch was richest in iron at the height of the spring diatom outburst, with 2·3 mg from each cubic metre of water filtered. During the summer with animals paramount, an average plankton iron content of about 0·5 mg per cubic metre was maintained.

When the plants are eaten by herbivores, the excess of iron over requirements is excreted in faecal pellets and becomes available more or less quickly for a second growth cycle.

VII—References

Armstrong, W. D. (1933). 'Ind. Eng. Chem.,' Anal. Ed., vol. **5**, p. 300.

Atkins, W. R. G. (1923). 'Trans. Faraday Soc.,' vol. **18**, p. 310.

Atwater, W. D. (1892). 'Rep. Commissioner for 1888, U.S. Comm. Fish,' Washington, No. 16, p. 763, citing Kostytscheff, P., 'J. Rural Economy and Forestry' (Russia), vol. **144**, Part II.

Bodländer, G. (1898). 'Z. phys. Chem.,' vol. **27**, p. 66.

Braarud, T., and Klem, A. (1931). 'Hvalrådets Skr.,' No. 1, p. 46.

Brandt, K., and Raben, E. (1920). 'Wiss. Meeresuntersuch.,' Kiel, vol. **19**, p. 175.

Britton, H. T. S. (1925, *a*). 'J. Chem. Soc.,' vol. **127**, p. 2110.

—— (1925, *b*). 'J. Chem. Soc.,' vol. **127**, p. 2148.

Cooper, L. H. N. (1933). 'J. Mar. Biol. Ass.,' vol. **18**, p. 677.

—— (1934). 'J. Mar. Biol. Ass.,' vol. **19**, p. 755.

Czapek, F. (1920). "Biochemie der Pflanze," vol. **2**, p. 356, Jena.

Elder, L. W. (1930). 'Trans. Amer. Electrochem. Soc.,' vol. **57**, p. 383, cited from Randall and Frandsen.

Feigl, F., and Hamburg, H. (1931). 'Z. anal. Chem.,' vol. **86**, p. 7.

Gran, H. H. (1931). 'Rapp. Cons. Explor. Mer,' vol. **75**, p. 37.

Gran, H H. (1933) 'Biol Bull,' vol. **64**, p 159.

Greef, A (1913) 'Ber deuts chem Ges,' vol **46**, p 2511

Harvey, H W (1925) 'J Mar Biol. Ass,' vol. **13**, p. 953

—— (1933) 'J Mar Biol Ass,' vol. **19**, p 253

Harvey, H W, Cooper, L H N, Lebour, M. V, and Russell, F. S (1935). 'J Mar Biol Ass,' vol **20**, p 407

Hill, R (1930) 'Proc Roy Soc,' B, vol **107**, p. 205

Hopkins, E F (1930) 'Bot Gaz,' vol **89**, p. 209

Jellinek and Gordon (1924). 'Z phys Chem,' vol **112**, p. 235, cited by Britton (1925, *b*)

Krassa, P (1909) 'Z Elektrochem,' vol **15**, p. 490, cited by Randall and Frandsen

Lamb, A B (1910) 'J Amer Chem Soc,' vol. **32**, p 1214, cited by Randall and Frandsen

McCance, R A, and Shipp, H M (1933) 'Rep Med Res. Council, Lond,' Spec Ser No 187

Morgan, G T, and Burstall, F H (1932) 'J Chem Soc,' p. 20.

Muller, E (1908) 'Z Elektrochem,' vol **14**, p 77

—— (1917) "Das Eisen und seine Verbindungen," Dresden and Leipzig

Muller, H (1933) 'Mikrochem,' vol **12**, p 307

Orton, J H (1923) 'Fish Invest,' Series 2, vol **6**, No 3, p. 166.

Patten and Mains (1920) 'J Ass Official Agric Chem.,' vol **4**, p. 233 Cited from Atkins (1923)

Randall, M, and Frandsen, M (1932) 'J Amer Chem Soc,' vol **54**, p 40

Schrager, B (1929) 'Coll Czechoslovak Chem Commun,' vol. **1**, p 275

Shipley and McHaffie (1924). 'Can. Chem Met,' vol. **8**, p. 121, cited from Randall and Frandsen

Sjostedt, G. (1921) 'Bot. Notiser,' p. 101, 'Bot. Abst,' vol. **13**, p 227 (1924)

Sweeney, W J (1928) 'Trans Amer. Electrochem Soc.,' vol. **53**, p 317, cited from Randall and Frandsen

Thompson, T G, and Bremner, R. W (1935, *a*) 'J Cons Explor Mer.,' vol. **10**, p 33

—— (1935, *b*) *Ibid*, p 39

Thompson, T G, Bremner, R W., and Jamieson, I. M. (1932) 'Ind. Eng Chem,' Anal, Edn, vol. **4**, p 288; 'Univ Washington Publ in Oceanography,' Suppl Series, No 4, pp. 1–7.

Thompson, T G, and Robinson, R J (1932) "Physics of the Earth," V. Oceanography, chap V, Washington, National Research Council.

Thompson, T G, and Taylor, H. J. (1933). 'Ind. Eng. Chem,' Anal. Ed, vol **5**, p. 87

Wattenberg, H (1927) 'Z Ges Erdkunde,' No. 5/6, p 308.

Whitman, W G, Russell, R P, and Davis, G H B. (1925) 'J Amer. Chem Soc.,' vol **47**, p 70.

Wilcox, L V (1934). 'Ind Eng Chem.,' Anal. Ed, vol **6**, p 167.

FIG 1—Annular "garter" of reticular tissue

(*Facing p* 430)

547.962.9 541 183 2

The Swelling of Structured Proteins

The Influence of the Reticular Tissue on the Swelling of Collagen in Water and Hydrochloric Acid

By Dorothy Jordan-Lloyd and R. H. Marriott

(From the Laboratories of the British Leather Manufacturers' Research Association)

(*Communicated by R. H Pickard, F R.S —Received June* 14, 1935)

[Plate 26]

Previous observations on the swelling of fibrous tissues and fibres (Kaye and Jordan-Lloyd, 1924, *a*, Marriott, 1932; Jordan-Lloyd, Marriott, and Pleass, 1933. Jordan-Lloyd and Marriott, 1933, 1934) have shown that the swelling of biological material is largely influenced by its structure Even a fibre that is histologically structureless, as, for example, a fibre of silk-fibroin (silk-gut), can be shown to consist of a number of fine fibrils held together by lateral cohesive forces Both with silk fibres and keratin fibres (horsehair), the evidence suggests that the structure of the fibre is maintained by what may be described as an equivalent of a system of internal girders, these girders being the lateral cohesive forces between the molecules. Keratin fibres (hairs) are of cellular origin, though a fibrous rather than a cellular structure exists in the hair shaft Collagen fibres are also of cellular origin, though in their fully developed form they are by no means such rigid structures as silk fibres or hairs, and swell much more freely It has been shown already that the swelling of collagen fibres, while woven together to form the tissue of the skin, depends both on the structure of the fibres and on the pattern of the weave, skin pieces where the weave is loose swelling more freely under the same conditions than skin pieces where the weave is more compact (Kaye and Jordan-Lloyd, 1924). Moreover, it has also been shown that if the fibres were invested with a film of coagulated protein, swelling was less than under fresh conditions. Swelling is, therefore, also a function of the external stresses exerted on the fibre, a generalization confirmed by Marriott (1932) in a study of the behaviour of single collagen fibre bundles teased out of the skin tissue.

Collagen fibres cannot be obtained free from all other tissues They are invested and held together throughout the skin by another fibrous protein, the reticular tissue (Kaye, 1929, Kuntzel and Prakke, 1933) This tissue forms the equivalent of a system of external girders round the actual

collagen fibres and influences their behaviour Collagen fibres from skin which were used in the earlier work are not a suitable material for the study of the influence of the reticular tissue on the swelling of collagen owing to the difficulty of teasing out a good supply of undamaged, suitable single fibres in a reasonably short space of time from fresh skin For this reason the tendons from rats' tails were selected for study. Previous work on the swelling of tendons has been recorded by Kuntzel (1926) and of their structure by Kuntzel and Prakke (1933).

In the present work tendons from young rats (six weeks old) and an adult rat were compared, since there was every reason to suppose that the proportion of reticular tissue to collagen proper would differ in the two ages.

The experimental results showed in a very striking way that in the young rats the reticular tissue round each collagen fibre is either greater in amount or else tougher than in the adult rats, so that it resists the swelling forces exerted by the tendon Even where the swelling force is at a maximum (about p_H 3 0) the encircling sheath is still strong enough to resist the swelling pressure, but if the sheath becomes damaged mechanically, then it tears and rolls back into the form of annular garters The strongly swollen collagen is very fragile, and if the tendon be shaken, it readily breaks up and disperses, leaving the reticular tissue as a complete ring, fig 1, Plate 26 Thus, the amount of swelling obtained for any tendon depends on whether and to what extent the reticular tissue is ruptured. It will be shown later that acid solutions with $p_H < 2$ 0 weaken the reticular tissue and allow it to give way under the forces of swelling The moment at which the reticular tissue gives way appears to vary somewhat with each individual tendon, a state of affairs that was, unfortunately, only realized at a late stage of the work and which from time to time is the cause of wildly aberrant results (*see* fig 4).

In fig. 4 are shown six pieces of tendon—three adult and three young—all swollen in the same experimental vessel at p_H 3 8. It is clear from the camera sketches that the adult tendons as a group, *a*, *b*, and *c*, are more swollen, the young, especially *d* and *f*, being smoothly held in the reticular sheaths Of the six experimental pieces, however, *c* from the adult group and *e* from the " young " group can be seen to have become conspicuously swollen on account of the giving way of the reticular tissue

Experimental Methods and Results

The tendons were pulled away from the tendon sheath and cut into convenient lengths. The tendons were kept in water with toluene as an antiseptic until required for use and were never allowed to become dry at

any stage of the experiment, since previous work has shown that drying alters the properties of collagen fibres. Initial and final dimensions were measured by means of a projection camera the scale of magnification of which could be controlled. Volumes were calculated from measurements of length and average measurements of width (5–10 taken for every fibre), assuming that the fibres were perfect cylinders under all conditions.

Only swelling in water and hydrochloric acid was investigated, but sufficient evidence was obtained to prove the influence of the reticular tissue. Unfortunately, circumstances arose which rendered it impossible to carry the experiments into a wider field.

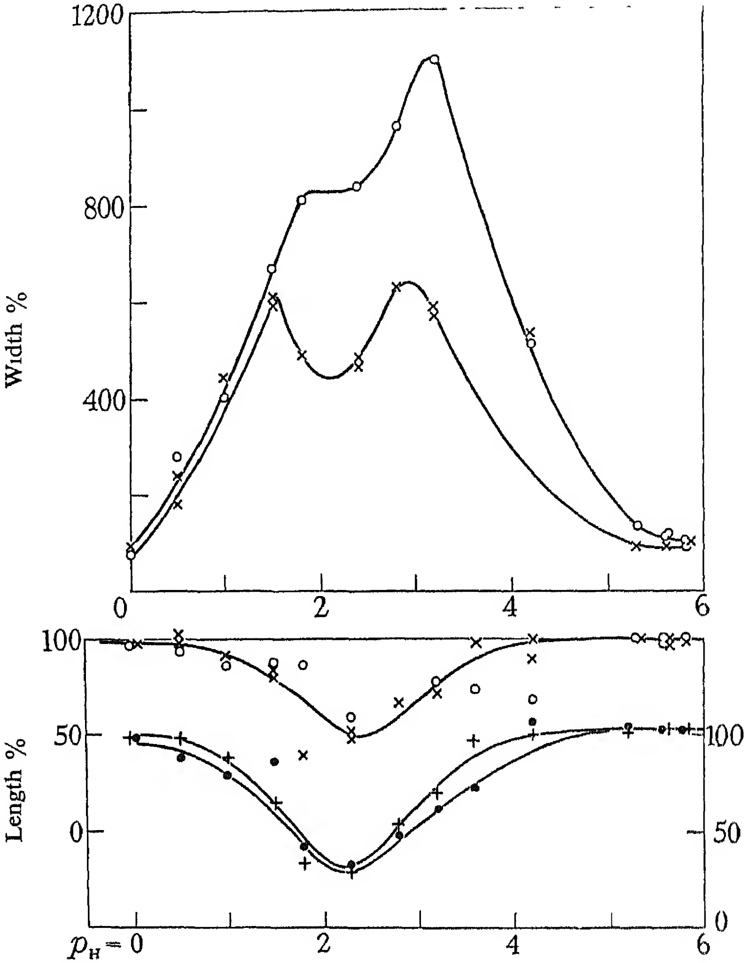
Curves showing the swelling over a p_H range of 0 — 5.8 are shown in figs. 2, 3, and 4. Experimental results for the tendons of adult rats are shown as open or solid dots and for those of young rats as sloping or upright crosses. One series of experiments appears in all three figures, a second as length measurements in fig. 3 only. Measurements of length and width after 24 hours' immersion in the experimental fluid are given in fig. 4. These represent equilibrium figures; volume measurements have been calculated, fig. 4. It will be noticed that increasing diameter is accompanied by diminishing length. The p_H of maximum increase of diameter is, however, not quite the same as that of maximum decrease in length. This confirms Marriott's previous observation on collagen fibres from fresh ox-hides that, owing to the decreasing length with increasing width, maximum volume occurs nearer neutrality than maximum width.

A series of sketches of tendons before and after swelling is shown in fig. 5. The rings of reticular tissue can be clearly seen. The irregular nature of the swelling in some cases shows that the tensile strength of the reticular tissue may vary even in the same tendon.

Discussion

In many previous swelling experiments with collagen fibres (Kaye and Jordan-Lloyd, 1924, *a*, *b*; Jordan-Lloyd and Marriott, 1933, 1934), it had always been noticed that with acid swelling a well-marked maximum, similar to that so familiar in gelatin gels, was always found, but that the descending branch of the curve never came down smoothly but always showed a notch at about p_H 2, in other words, swelling in collagen fibres at p_H values < 2 was relatively greater than at values > 2 when compared with gelatin. Since it was observed that under these conditions fibre bundles from the skin showed an increased splitting up into the component fibres and fibrils, the suggestion was put forward that at about p_H 2 there was a weakening of certain structures in the fibre bundle. This suggestion is

fully confirmed by the present work The reticular tissue restrains the swelling of the fibres in acid solutions Where the tissue is weak (as shown by mechanical rupture), swelling is greater than where it is strong Visual examination shows that in young rat tendons the reticular tissue is stronger



FIGS 2 and 3—The upper figure is fig 2, and the lower fig 3. o and • tendons of adult rats, × and + tendons of young rats

than in adult rat tendons Therefore, with a p_H range of 6–2, tendons from young rats swell less than those from adults * At p_H values < 2, however,

* [*Note added in proof*, *September* 19, 1935—Since going to press, a paper has been read by Professor Leplat at the Brussels Congress of the International Society of Leather Trades' Chemists in which he stated that tendons from the tail of the adult cat showed swelling in acid solutions but that those from the newly born kitten showed no swelling under the same conditions on account of the restraining influence of the reticular tissue.]

the reticular tissue is itself weakened by the acid, and where this has occurred the swelling of both young and old tendons is of the same order.

There is great contrast between adult and young tendons at p_H 3 3 on the ascending branch of the swelling curve, and lesser contrast at p_H 1 5 close to the notch in the curve, while there is absence of contrast at p_H 1 0 on the descending branch

A characteristic of the swelling of collagen fibres is the shortening which occurs along with lateral expansion It has already been shown that the two processes run roughly parallel though they do not coincide There

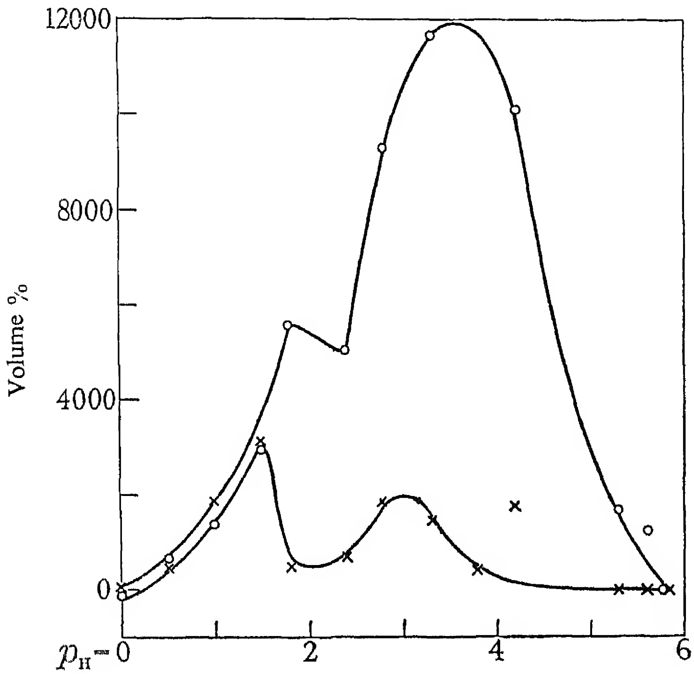


FIG 4—o tendons of adult rats, × tendons of young rats

appears to be no correlation between the degree of shortening and the age of rat tendons. Shortening may therefore be attributed to the properties of the collagen and to be uninfluenced by the reticular tissue

Shortening of the collagen fibre could be brought about either by a contraction of the "back-bones" of the molecules that build up the fibrils, such as Astbury and Woods have shown to occur in the transformation of β-(stretched) keratin to α-keratin and α-keratin to the "super-contracted" form, or it could be brought about by the effect on a ladder-like structure with flexible sides but rigid non-elastic rungs of intrusion of the fluid drawn in round the osmotic centres during swelling. This would lead to a bulg-

ing and shortening of the "sides" of the ladder (Jordan-Lloyd, 1932, Kuntzel, 1934) Since Astbury and Atkin (1933) have shown that the "backbone" of the collagen molecule already shows considerable shortening (length of unit = 2·8 A as compared with 3 5 A in the fully extended unit of silk fibroin), the latter explanation appears more probable.

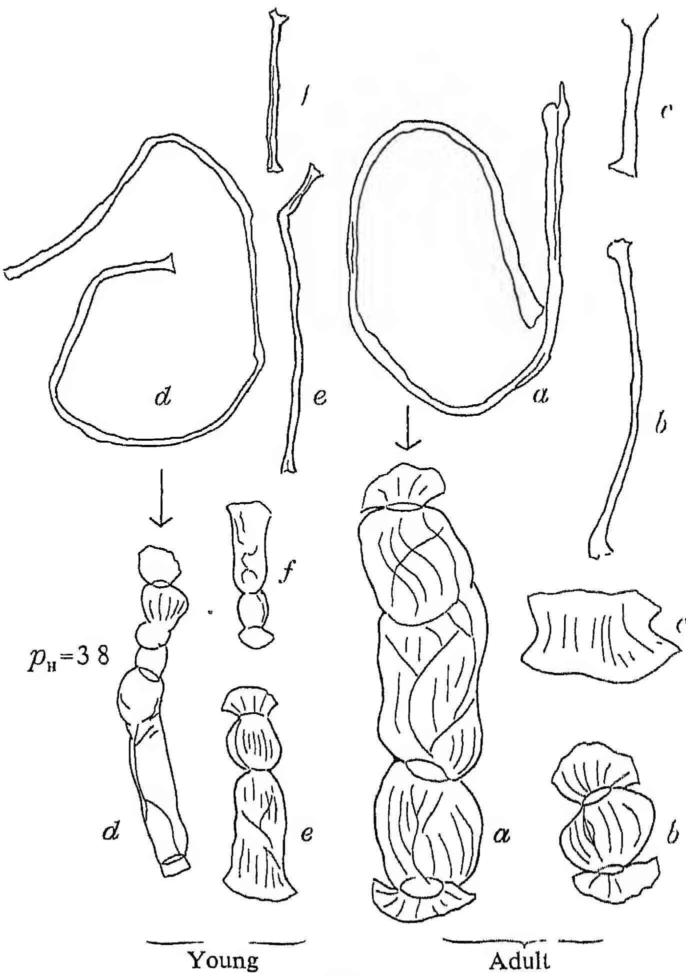


FIG 5

The experiments on the young and old tendons show clearly that any interpretation of the swelling curves of biological structures must take into account biological factors as well as physico-chemical ones. It is unfortunate that the alkaline range was not covered by the experiments. Gelatin shows a clear swelling maximum at p_H 10–11, but a long series of experiments with collagen fibres has never revealed a clear-cut alkaline maximum.

At p_{H} values > 11 the swelling curve runs an irregular course until it finally turns upwards as hydrolysis sets in A weakening of the reticular tissue at a p_{H} value lying close to that of maximum alkaline swelling might well account both for the absence of the maximum and the irregular nature of the experimental data

Any attempt to explain the various maxima and minima on the swelling curves of structured proteins on pure physico-chemical lines is obviously foredoomed to failure

Summary

Collagen fibres from the tendons of rats' tails are always invested with a fine reticular sheath, the reticular tissue The swelling of the collagen fibres is accompanied by a mechanical rupture of the reticular sheaths, which roll back to form reticular "garters" or rings Young tendons have either a greater amount of reticular tissue or a tougher variety and therefore, in general, swell less than old tendons In hydrochloric acid solutions of $p_{\mathrm{H}} < 2$, the acid weakens the reticular tissue In these solutions, young and adult tendons swell similarly, and to a greater extent than would have been anticipated from their behaviour in more dilute solutions

Collagen fibres contract in length while swelling This contraction is similar in adult and young tendons and is due to a property of the collagen

References

Astbury and Atkin (1933) 'Nature,' vol **132**, p 348
Astbury and Woods (1933) 'Phil Trans,' A, vol **232**, p 333
Kaye and Jordan-Lloyd (1924, *b*) 'Biochem J,' vol **18**, p 1043
Kaye and Jordan-Lloyd, (1924, *a*) 'Proc Roy Soc,' B, vol **96**, p 293
Kaye (1929) 'J Soc Leather Tr Chem,' vol **13**, p 73
Kuntzel (1926) 'Kolloid Z,' vol **40**, p 264
Kuntzel (1934) 'Collegium,' No **765**, p 1
Kuntzel and Prakke (1933) 'Biochem, Z,' vol **267**, p 243
Jordan-Lloyd (1932) 'J. Soc Chem Ind,' vol **51**, p 141T
Jordan-Lloyd and Marriott (1933) 'Trans Faraday Soc,' vol. **29**, p 1229
—— (1934) 'Trans Faraday Soc,' vol **30**, p 944
Jordan-Lloyd, Marriott, and Pleass (1933) 'Trans Faraday Soc,' vol **29**, p 554
Marriott (1932) 'Biochem J,' vol **26**, p 46

615.711 71

The Pharmacology of Ergometrine

By G. L Brown and Sir Henry Dale, F R.S

(From the National Institute for Medical Research, Hampstead, London, N.W.3)

(*Received September 2, 1935*)

I—Introductory

The action of ergot on the human uterus has been a matter of popular tradition for more than three centuries, and for more than a century it has found a recognized application in obstetrical practice Until recent years, the preparation most commonly employed was an extract made by macerating the drug with water , and this was usually given by the mouth. This widely used watery extract, however, usually contained no significant amount of any of the six or seven alkaloids specific to ergot, which had been isolated from it at wide intervals over a period of about 60 years (ergotinine, ergotoxine, ergotamine, ergotaminine, ψ-ergotinine, sensibamine, and ergoclavine). The same was true of a number of the proprietary preparations which had been widely used in practice. Further, while several of those alkaloids (ergotoxine, ergotamine, sensibamine, ergoclavine) have a powerful, highly characteristic, and identical pharmacological activity when given by parenteral injection, including a stimulant action on the uterus, their actions are relatively weak and irregular when they are given by the mouth The proteinogenous amines tyramine and histamine, which had been found to occur in extracts of ergot, whether as products of the metabolism of the fungus itself or of putrefactive changes occurring during the extraction, also have different and well-known pharmacological actions when they are injected into the blood-stream, including stimulant actions of different types on the uterus ; but they are again almost without action when given by the mouth in doses having relevance to the activity of ergot extracts. The anomalous position had therefore arisen that the active substances known to occur in ergot were either not present in extracts of the drug which had been used widely and with satisfaction for many years, or, if present, were without significant action when administered by the method commonly employed.

This anomaly was completely removed when Moir (1932) showed that the old-fashioned watery extract of ergot, containing none of the alkaloids hitherto known, when administered by the mouth to women in the puerperium, promptly evoked a vigorous rhythmic activity of the previously

quiescent uterus, as shown by a simple mechanical record, and when Dudley, whose chemical work was controlled at each stage by Moir's clinical records, isolated a new alkaloid, ergometrine (Dudley and Moir, 1935), much simpler in structure than, though evidently related to, the previously known ergot alkaloids, soluble in water to some extent as a free base, and giving salts which are readily soluble in water, and easily absorbed from the alimentary canal.

It is difficult at first sight to explain the fact that the many experienced investigators who, over so many years, have sought for the active principle of ergot have failed to obtain this alkaloid An explanation may suggest itself when we have described its action, as shown by ordinary pharmacological methods When once it had been identified, it was seen to be the easiest of all the ergot alkaloids to extract and purify This circumstance, together with the interest aroused by the removal of a long-standing anomaly, has led to the appearance, within three months from the date of Dudley and Moir's publication, of three other claims to isolation and identification of a water-soluble alkaloid of ergot, acting with administration by the mouth As Dudley has shown in the succeeding paper, there is no good reason for supposing that the substance obtained by any of these claimants is anything other than ergometrine, but they have put forward three different names for it (Ergotocin, Ergobasine, and Ergostetrine), and pharmacological descriptions of the action of "Ergobasine" (Rothlin, 1935) and of "Ergotocin" (Davis, Adair, Chen, and Swanson, 1935) have already appeared Where these publications deal with experiments of the same kind as those which we have made with ergometrine, the results support the chemical evidence for the identity with ergometrine of the alkaloids thus variously named.

The ergometrine which we have used in these experiments has been from several samples, one prepared by Dr Dudley himself, and others supplied for investigation by a number of British pharmaceutical manufacturers (Boots Pure Drug Co., British Drug Houses, Burroughs Wellcome & Co, Allen & Hanburys, May & Baker, Duncan & Flockhart) The pure crystalline alkaloid (M.P 161—162° C) has been weighed out as required, dissolved in about 1 cc of N/100 H_2SO_4 to each milligramme of alkaloid, and then made up with water or physiological saline to any required dilution A solution so prepared is neutral or faintly alkaline Dosage is indicated in weight of pure alkaloid

II—General Toxicity

On the different species on which we have examined its action, ergometrine has produced symptoms recalling some of those produced by the

earlier-known ergot alkaloids (ergotoxine, ergotamine, etc.), but usually less severe with equal dosage A strictly quantitative comparison of its toxicity for any species with that of any of these other alkaloids would have required large numbers of animals, in order to determine the doses producing identical percentage mortalities We have not attempted this but have confined our attention to as accurate comparison as possible of the general symptoms produced by non-lethal doses. Rothlin gives (for ergobasine) the lethal dose for mice as 0·145 mg/gm, and for ergotamine as 0 045 mg/gm for the same species. His figures for rodents in general show a toxicity of ergotamine about 3 to 3½ times as great as that of ergobasine Our own comparisons of ergometrine with ergotoxine, on a basis of severity of symptoms, are compatible with this finding ; though it is a little puzzling that Rothlin's lethal dose for ergobasine on mice did not, in our experiments, cause any fatalities even with ergotoxine. It is difficult, however, to appraise the meaning of this apparent discrepancy, since Rothlin's table would seem also to require doses of 15 grams of ergobasine (7 5 mg/gm) and 4 grams (2 mg/gm) of ergotamine to kill an average rabbit of 2 kg We are bound to suspect some textual errors.

(*a*)—Mice

Ergometrine—Mice of 13 to 17 g body weight received injections of 0·01 to 0·1 mg of ergometrine per gm by intravenous injection The only symptoms seen in any were slight exophthalmos and erection of the hairs, suggesting a general stimulation of sympathetic nerves, probably of central origin. The symptoms became more obvious as the dosage was increased, but were in no case pronounced. All the mice recovered rapidly, and there were no deaths or after-effects.

Ergotoxine—Groups of mice from the same batch received similar injections of ergotoxine ethanesulphonate, over the same range of doses, calculated in free alkaloid. With every dose the symptoms were much more pronounced than those produced by ergometrine, the mice showing tremors, extreme excitability, and a pronounced weakness of the hind limbs, with a tendency to backward running. Even 0 15 mg/gm, however, produced no fatalities in our series, all the mice being apparently normal on the following day

Ergine—Through the kindness of Dr S. Smith, of the Wellcome Chemical Works, we had the opportunity of making a few experiments with this artificial cleavage product from ergot alkaloids. Ergine is the simple amide of lysergic acid, of which ergometrine is the hydroxyiso-

propylamide, according to Jacobs and Craig (1935). Ergine, in our few experiments, has shown a much greater immediate toxicity than any of the ergot alkaloids, and practically none of their specific activities on the uterus and other organs In doses of 0 01 to 0 04 mg/gm, given intravenously to mice, it produced urgent symptoms, with trembling, weakness of the legs, and exophthalmos ; and in a dose of 0 08 mg/gm it was rapidly fatal

(*b*)—RABBIT

Ergometrine, when injected by the ear vein in doses of 1 8 to 2 8 mg per kg, produced in the rabbit symptoms showing a general resemblance to those following similar doses of the more complex ergot alkaloids The pupils show immediately a wide dilatation, and there is some exophthalmos The ears become pale and cold with intense vaso-constriction. The animal is restless and excitable, and the respiratory rate gradually increases to an intense tachypnoea In one experiment, 30 minutes after injection of 2 8 mg per kg, the heart-beat was at 300 p m. and the respiration at 333 p m. This severe hyperpnoea shakes the whole animal, and bubbling sounds are audible at some distance There is some weakness, progression being by crawling rather than by the normal hopping gait The symptoms pass away rather rapidly , the hyperpnoea and mydriasis are declining after two hours, and at the end of three hours some rapidity of the breathing is the only overt symptom

The whole effect, with excitement, trembling, hyperpnoea, and signs of sympathetic stimulation, was reminiscent of the action of such a central stimulant as β-tetrahydronaphthylamine Like this substance, again, and like the more complex ergot alkaloids, ergometrine causes a rise of internal body temperature. In a rabbit which received 1 8 mg of ergometrine per kg by intravenous injection, the rectal temperature, recorded by a thermocouple, rose steadily from the initial level of 39°, to reach a maximum of 41·5° in 70 minutes, thereafter falling steadily again to reach 39·8° in 190 minutes after the injection Davis *et al.* (1935) report no increase of metabolism in the rat after doses of 1 to 3 mg (Ergotocin) per kg, but definite increases after 5 or 10 mg per kg. Probably the rat is less sensitive than the rabbit, in which such a rise of temperature as we observed would be expected to involve a noteworthy increase of metabolism Rothlin observed a rise of temperature in the rabbit with as little as 0·1 mg per kg (Ergobasine).

(*c*)—CAT

Experience with the more complex ergot alkaloids had shown that the normal cat exhibits particularly well-marked and characteristic symptoms

when these are injected. (*Cf.* Barger and Dale (1907, 1909), Dale and Spiro (1922))

Intravenous Injection—Ergometrine was given to a number of cats by intravenous injection, in doses of 2 to 2 5 mg per kg The effects were constant and characteristic, and may be said to include the excitatory phase seen in the action of the more complex alkaloids The cats were secured on the table for the injection by strings round the wrists and ankles, the head being held by an assistant The internal saphena vein was exposed for injection, by a small incision just above the knee, made under local anaesthesia with cocaine. Almost as soon as the injection has been completed the onset of the effect is visible, in the rapid dilatation of the pupils and erection of the hairs on the tail. By the time the cat can be released, it shows already some inco-ordination and tendency to sprawl, and a pronounced excitability and " sham rage," starting, snarling, spitting, baring its teeth and extending its claws, in response to visual or auditory stimuli of any kind, but making no purposive movements of attack or defence ; so that it can be lifted without danger of bite or scratch, in spite of its apparent threatening reaction to the approaching hand The pupils have now dilated to a maximal extent, and exophthalmos has become so pronounced that blinking fails to cover the cornea, which often becomes dry and shows small abrasions before the effect has passed away. Erection of the hair on the head, back, and tail is also definite and persistent Weakness of the limbs increases, the animal crouching with the joints flexed and the fore feet widely apart. When set free to walk it crawls slowly, with the belly touching the floor, and with a tendency to remain for some seconds in a position of uncompleted movement. The weakness and inco-ordination are central, however ; for a sudden noise will often cause the cat, at this stage, to make a clumsy vertical spring of a foot or more from the floor These effects resemble strongly those seen in the earlier excitatory phase of the action of ergotoxine and ergotamine, in similar or smaller doses These alkaloids, however, show additional effects. The initial mydriasis, never so pronounced as that caused by ergometrine, soon gives way to an intense miosis, which persists for many hours The weakness is more pronounced, and affects the hind limbs earlier, so that the animal, if disturbed, thrusts itself backwards along the floor The weakness ultimately becomes general, the animal then often lying flaccid on its side and the anal sphincter often becomes incompetent. The appearance of a cat when the symptoms of poisoning by ergotoxine or ergotamine are fully developed is highly characteristic ; it lies inert and half-conscious, with the pupils constricted to the narrowest slits, even

in a dark corner, and reacts but feebly, though still with signs of sham rage when disturbed. In a cat which has received ergometrine these later effects are entirely missing. The excitability slowly subsides, and the movements become gradually stronger and more co-ordinate. The mydriasis and exophthalmos persist longer, and are distinctly visible, though no longer maximal, five hours after the injection On the following day the animal is again completely normal As in the rabbit, these effects of ergometrine in the cat, with their prominent component of sympathetic stimulation, recall the action of β-tetrahydronaphthylamine and, to some extent, that of morphine on the cat. For reasons which will appear later, we were led to suspect that ergometrine had some peripheral sympathomimetic action ; and we accordingly made a few experiments to determine how far the symptoms of intense, general sympathetic stimulation, seen in the unanaesthetized cat, were due to this, and how far they were to be attributed to excitement of the nerve centres.

1. Removal of the superior cervical ganglia aseptically under ether, a few days previously, produced a definite sensitization of the corresponding eye to the mydriatic effect of ergometrine given intravenously The pupil on the operated side dilated more rapidly and to an even wider maximum than that on the intact side This might represent a sensitization to the direct action of ergometrine itself, or to adrenaline secreted in response to its central action

2. If, in addition, the splanchnic nerves were cut on both sides, or one splanchnic nerve cut and the opposite suprarenal gland removed, the denervated eye no longer showed a preferential reaction to the injection of ergometrine It still showed a distinct dilatation of the pupil and withdrawal of the nictitating membrane; but the effect was now slower and less complete than on the intact side. The relatively weak peripheral sympathomimetic effect of ergometrine accordingly plays at most a minor part in the intense effects of sympathetic stimulation shown by the eye Also the discharge of adrenaline, made evident by the denervated pupil when the splanchnics are intact, is due to a central action of ergometrine and is eliminated when these nerves are cut

3 A similar conclusion could be drawn from another experiment in which, by preliminary aseptic operation under ether, we cut the splanchnic nerves on both sides, and removed on both sides the last lumbar and first sacral sympathetic ganglia ; thus decentralizing the ganglia which innervate the pilomotors of the tail. A

fortnight later, this cat, receiving the usual intravenous dose of ergometrine, showed all the characteristic features of its action, except that the pilomotor effect, evident as usual on the top of the head and down the middle of the back, stopped short at the root of the tail, on which the hair lay flat right down to the tip.

4 When the suprarenal glands were both removed, by two separate aseptic operations with an interval of some weeks between them, injection of ergometrine still caused some mydriasis of the pupil on the side from which the superior cervical ganglion had been removed, though less, of course, than on the intact side. This remnant of mydriatic effect, therefore, is due to a direct effect on the eye, or to depressant action on the nucleus of the third nerve. Its occurrence when the suprarenal glands were only denervated was accordingly not due to a direct stimulation of the suprarenal medulla by ergometrine , indeed, we shall later find direct evidence that ergometrine has no significant action of that kind. We observed dilatation of the pupil of an isolated cat's iris, when ergometrine was added to the saline bath. Davis, Adair, Chen, and Swanson (1935) report a mydriatic effect of ergotocin with direct application to the eye of the rabbit.

The two experiments, made on cats thus deprived of both suprarenals, brought to light another fact of some interest, which we record without being able to offer any satisfactory explanation. In normal cats, though the symptoms produced by the dose of ergometrine injected have always been striking, recovery has always been rapid. In the cats without suprarenals, though neither had begun to show definite symptoms due to the deprivation, the same dose of ergometrine caused initial symptoms of excitation; but these were early followed by a depressor phase, which ended in death 1 to 3 hours after the injection. The details of the second such experiment may be given

Cat. 2 *kg*

12 6 35—Aseptic removal of right suprarenal under ether.

29.7 35—Aseptic removal, under ether, of left suprarenal and right sup. cerv ganglion.

31.7.35—Cat in good condition No signs yet of cortical deficiency.
11.15 a m 5 mg ergometrine i.v. Immediate dilatation of both pupils, right less than left Both nictitating membranes retracted

and eyes protruded. Erection of hair on back and tail ; pronounced " sham rage."

11 17 a.m. Weakness of limbs. Cat now lies on its side.

11.20 a m Pulse 135 p m

11 35 a m Lies on side Knee-jerks exaggerated All limbs extended but not rigid

11 54 a.m Pulse 114 p m. Still snarls and spits on being touched

11 55 a.m Limbs extended and definitely rigid Some opisthotonus.

11 59 a.m. Pulse 106 p.m.

12 00 noon Apparently unconscious Rigidity of " decerebrate " type. Conjunctival and tendon reflexes brisk

12 06 p m Pulse 100 p m Rect. temp 37·5°. Respiration regular, but with occasional deep sighs

12 20 p m. Pulse 90 p.m

12.25 p m Conjunctival reflex absent. Knee-jerks still brisk Rigidity of limbs still pronounced

1 00 p.m Respiration becoming progressively slow and weak.

2 00 p m Cat dead

In neither of these cats was any cause of death found post-mortem, or anything, apart from the clean adrenalectomy, to account for the fact that, after an initial reaction to ergometrine of the type seen in normal cats, they lapsed into this progressive and fatal depression.

On two occasions intravenous injections of ergometrine were given to cats which were pregnant, the dose in each case being 5 mg. One of these was at a late stage of gestation, and would almost certainly have given birth within a few days. It showed the usual symptoms, with a rather less pronounced excitement than some cats have shown. For two hours after the injection the uterus was palpated at intervals through the abdominal wall ; no continuous contraction was present, the foetal parts being readily felt The cat was returned to its cage, and during the night gave birth to two normal, living kittens with no signs of prematurity They were nursed by the mother, and their eyes opened 10 days after birth. In the other cat, which had apparently completed about two-thirds of the normal gestation, the pregnancy was not suspected when the injection of ergometrine was given. The usual symptoms followed, and at the end of 5 hours, when the excitement had subsided, bleeding from the vulva was observed. The onset of abortion was then suspected, and during the night the cat gave birth to four kittens, two in unbroken membranes, and

these were found dead in the cage on the following morning Their appearance indicated about the sixth week of development The mother seemed to be practically normal and showed no after-effects

In the one case, therefore, this toxic dose of ergometrine accelerated parturition, or at least caused neither delay nor injury to the offspring ; in the other case it caused, apparently, a precipitate expulsion of immature foetuses without any injury to the mother. These effects contrast with those observed by Thompson (1935) with his "alkaloid X," in which doses as low as 1 mg given by mouth caused death of both foetuses and mother. Such a result, we believe, cannot have been due to ergometrine alone.

Administration by Mouth—With oral administration of 5 mg to a cat we observed dilatation of the pupils, retraction of the nictitating membrane, and slight exophthalmos in 5 minutes. The animal was quiet, and showed none of the symptoms of sham rage seen after injection. Salivation was profuse ; but this may probably be attributed to the bitter taste of the alkaloid We found it impossible to induce a cat to swallow 5 mg dissolved in 1 cc of water. It was taken after dilution to 20 cc, but followed by salivation which persisted. 10 mg similarly administered in 50 cc to another cat was promptly vomited, and no other symptoms of its action appeared It is apparent that these widely hypertherapeutic doses are irritating to the stomach.

Hypodermic Injection—The vomiting with oral administration, however, is not entirely due to a direct irritation of the gastric mucosa, since it occurs when ergometrine is injected hypodermically. We have never seen vomiting after intravenous injections. Apart from its occurrence, the effects after hypodermic injection resemble those produced by similar doses given intravenously, except in their slowness of onset. Thus the typical "sham rage," which appears within a minute after intravenous injection, does not begin until about 30 minutes after hypodermic injection, and reaches its maximal development only after 45 minutes. Even slower in reaching their maximum after hypodermic injection are the mydriasis and exophthalmos, which appear so early after an intravenous injection.

With a smaller dose, such as 0·5 mg per kg, the effects are of the same kind as those following 2 to 2·5 mg per kg, but fall short of the maximum development With 0·2 mg per kg, the symptoms are perceptible but slight. For the sake of comparison, it may be mentioned that the largest dose recommended for use by injection in human therapeutics is about 0 005 mg per kg.

(*d*)—Dog

Only one experiment has been made on a dog The animal was a healthy male terrier, weighing 12·2 kg. 10 mg of ergometrine were given by injection into the external saphena vein The dog showed little immediate effect from the injection, but about 5 minutes after it had a short spell of excitability, in which it yelped and struggled if held by the collar. This soon passed, and during the next 3 hours the only symptoms observed were vomiting, which occurred thrice in the first hour after the injection, and a perceptible weakness, particularly of the hind legs

(*e*)—Fowl

The easily observed changes in the colour of the comb, and its liability to the gangrene produced by ergot, have made the domestic cock a familiar test-animal in investigations on ergot since the middle of the nineteenth century There was no doubt that ergotoxine and ergotamine produced gangrene (Barger and Dale, 1907, Dale and Spiro, 1922) , Rossler and Unna (1935) have recently demonstrated that sensibamine, in this or in other respects, is similar to the earlier known alkaloids , and, though Kreitmair (1934) does not record actual gangrene with ergoclavine, its action is in all other respects so closely equivalent to that of ergotoxine or ergotamine that it may be assumed that it would produce this effect also, if given in suitable doses at the proper intervals

There was one anomaly still to be explained, however Ergot given by the mouth to a cock caused a very deep cyanosis of the comb to appear within an hour of administration ; ergotoxine, on the other hand, when similarly administered in doses corresponding to the content of the ergot in alkaloids of this type, had a relatively slight, irregular, and delayed effect. There was no evidence that any of the alkaloids of this group had a more rapid and potent action on the cock's comb with oral administration. It was of interest, therefore, to see whether ergometrine had similar effects and would produce them when given by the mouth.

It can be said at once that the acute symptoms produced in a cock, when ergometrine is injected intramuscularly, are very similar to those seen with an equal dose of ergotoxine similarly injected Our impression was that the dyspnoea and other general symptoms produced by ergometrine were somewhat less pronounced than those produced by ergotoxine in equal doses. It is very difficult to be certain on such a point unless a large number of comparisons is made of the effects of both on the same birds; but this, while eliminating individual variation, forbids *simultaneous* comparison of effects which are, in any case, not susceptible of quantitative

record. We must be content, therefore, to record that ergometrine, in doses of 0 75 to 1 mg per kg, causes definite symptoms of inco-ordination and depression, with dyspnoea, staggering gait, drooping wings, salivation, and diarrhoea, qualitatively very similar to those produced by the other alkaloids, but probably less severe. It also produced the familiar colour-changes of the comb, beginning with circumocular pallor; pallor then spreading to the root of the comb and wattles, while the more peripheral portions became cyanotic ; and finally the whole comb and wattles becoming dusky purple in colour, cold to the touch, and showing areas of ischaemia following light pressure, which only slowly regained the general coloration This effect, when both were injected intramuscularly, was a little quicker in onset with ergometrine than with ergotoxine, but of about the same maximum intensity

A pronounced difference was to be seen, however, when two cocks of similar sensitiveness, thus injected with equal doses of ergometrine and ergotoxine respectively, were examined on the following morning. It was then seen that the bird which had received ergometrine had recovered completely, the comb being uniformly bright red and warm The bird which received ergotoxine, on the other hand, though the general symptoms had subsided, always showed, and continued on one or two successive days to show, a persistence of the cyanosis and coldness of the hinder end and digitations of the comb This difference between the two alkaloids was more clearly seen with larger doses, such as 10 to 20 mg injected into cocks of 3 kg. Even after such large injections, the effect of ergometrine, intense for the few succeeding hours, had almost completely vanished on the following day, while the action of ergotoxine on the comb was still very pronounced

The question naturally presented itself whether ergometrine, in addition to causing the acute cyanosis, would produce gangrene of the comb with repeated administration. We have made only one experiment on the point, but the answer seemed clear.

Two White Leghorn cocks were taken, which had earlier shown very similar immediate effects with ergometrine and ergotoxine, and which had very similar, bright red, healthy combs One weighing 2·6 kg was given by injection, on successive days, 15, 10, 10, 20, and 10 mg of ergometrine—65 mg in all Each injection was followed by the usual symptoms and cyanosis ; but on the mornings of the days following the first three injections no persistence of the action was perceptible, and on those following the last two injections only slight blueness and coldness of the hinder end and digitations could be detected. Even that was doubtfully more than could be accounted for by the cold spring nights On the second day

after the final injection the comb was again entirely red and warm, and remained so for the ensuing weeks of observation.

The second cock, weighing 3 1 kg, received only four injections of ergotoxine—15, 10, 10, and 20 mg—on successive days In this case, the immediate symptoms being similar to those produced by ergometrine, the cyanosis of the comb persisted between the injections and became progressively intensified, the comb becoming also, after the first injection, flaccid and shrunken. After the third injection (making 35 mg in all), dry gangrene of the hinder end and digitations of the comb was already apparent , this progressed after the subsequent injection, until finally about one-half of the comb, with all the digitations, was converted into a shrivelled, horny, black mass

On the basis of this comparison we should have concluded that ergometrine, while equally effective in producing the immediate cyanosis of the cock's comb, is free from the tendency to produce gangrene which is associated with the more persistent action of the more complex alkaloids. This conclusion was in agreement with Rothlin's statement concerning ergobasine, that "in the cock there is cyanosis of the comb and the wattles but never gangrene, even with large doses" Davis, Adair, Chen, and Swanson, however, injecting 0 5 mg of ergotocin into each of 6 cocks twice daily for 24 days, observed gangrene of the comb in 3 out of 6, occurring, if we rightly interpret the statement, 8, 5, and 15 days after the injections were finished. The total (24 mg) is much smaller than that which we gave, but its administration was spread over a longer period. This ergotocin used by Davis *et al*, assuming it to be the same alkaloid as ergometrine, was a rather less pure specimen, melting at 155°, as compared with 161–162° for ours. It is unlikely, however, that the effect described could be produced by traces of impurity. The only conclusion which we can safely draw is that ergometrine, as its more rapidly evanescent action would lead one to expect, has a denifitely smaller tendency to produce gangrene than ergotoxine and the other similarly acting alkaloids ; but that the production of gangrene even by pure ergometrine, if administered in appropriate dosage over a sufficient period, remains a possibility.

When ergometrine and ergotoxine are given to cocks by the mouth, being introduced in solution by a tube passed into the crop, the contrast between the effects of the two is striking. Ergometrine produces, with this method of administration the typical cyanosis, the beginning of which, with pallor of the skin round the eyes, can be detected within 5 minutes of the administration The maximal effect, which is comparable with that produced by the same dose with injection, is reached in half an

hour. As after injection of ergometrine, the effects of an oral dose subside from about the third hour and have completely disappeared by the next day When ergotoxine is given in this way its effect may be very slight in comparison with its effect when injected. In some cocks, however, ergotoxine given by mouth also produces the typical cyanosis of almost the same intensity as that caused by the same dose of ergometrine. The effects differ, however, in that the beginning of the ergotoxine action cannot be detected until 45 to 60 minutes after administration and that, like the effect of the same alkaloid when given by injection, the effect can still be detected on the following day. Ergotoxine, as might be expected from its larger molecule and poor solubility, is more slowly absorbed into and more slowly removed from the system than is ergometrine; and the differences of its effects on the cock from those of ergometrine, including the greater readiness with which ergotoxine produces gangrene, may all be reasonably attributed to these differences in physical properties

(*f*) Frog

Kobert had reported convulsions in frogs, resembling those produced by strychnine, as the result of injecting very small doses (1/32 mg) of his alkaloidal preparation Cornutine; but no subsequent observer had been able to observe anything like this action, either with preparations which should have corresponded to "Cornutine" or with the hitherto known alkaloids in pure form. It was of interest, therefore, to see whether the ergometrine had any such action It has been injected into the lymph-sacs of frogs in doses up to 0·05 mg per g, and has produced as its only effect a vague weakness, lethargy, and stiffness of movement, resembling that seen with ergotoxine and the other alkaloids.

III—Effects on Arterial Blood Pressure, etc.

These have been studied chiefly in the cat. In a few experiments on anaesthetized rabbits (urethane) ergometrine (0 01–0·5 mg) intravenously has always produced a small but definite rise of arterial pressure, with no depression of the respiratory centre, contrasting strongly with the large fall of arterial pressure, often terminating fatally, produced by an equal injection of ergotoxine on a rabbit similarly anaesthetized (*cf* Fig. 9). In the cat, on the other hand, we early observed a remarkable contrast which Davis, Adair, Chen, and Swanson have also noted, between the effect on a spinal cat, in which ergometrine produces a definite, though not very large, pressor effect, and that on a cat anaesthetized with ether

or chloralose, in which a similar dose of ergometrine produces a large fall of arterial pressure

Spinal Cat—The effect shown at A in fig. 1, as the result of injecting 10 mg of ergometrine intravenously into a spinal cat of 3 8 kg is an unusually pronounced and persistent one At B, 14 minutes after A, by which time the pressor effect had passed its maximum, a second injection of 15 mg of ergometrine caused no revival of the effect but, on the contrary, a fall of the pressure to about the original level This is the usual result in our experience of a second injection, even when the effect of the first is definitely pressor, as in fig 1

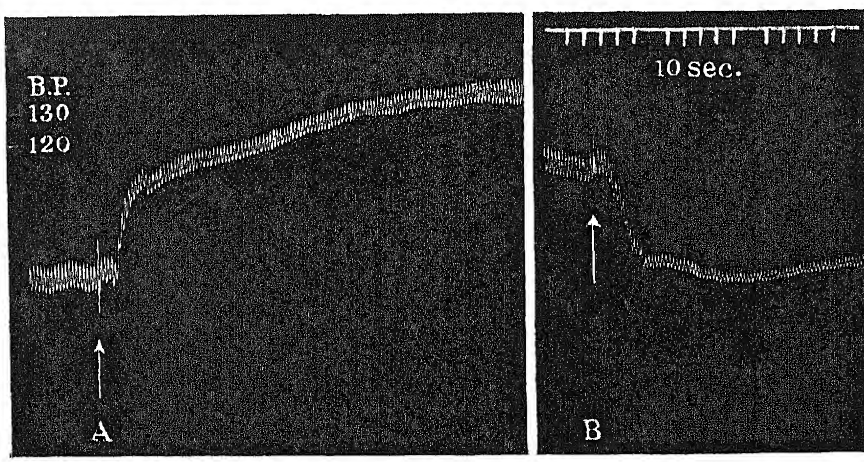


Fig. 1—Spinal Cat, 3 8 kg Blood pressure A, 10 mg ergometrine, B, 15 mg ergometrine.

Such a depressor effect of a second injection is often seen with ergotoxine, after the much more powerful pressor effect of a first injection of that alkaloid. It seemed possible accordingly that it might be connected with the paralytic action of ergotoxine on motor sympathomimetic actions, causing the well-known reversal of certain augmentor actions of adrenaline Rothlin states that this action is absent from the effects (of ergobasine), but Davis, Adair, Chen, and Swanson find that large doses (ergotocin) diminish the pressor response to adrenaline without reversing it This corresponds with our own experience

Fig. 2, from the same experiment as fig. 1, shows that the effect of adrenaline (20 γ) at A is much reduced by the injections of a total of 25 mg of ergometrine given between A and B , at B 40 γ and at C 100 γ of adrenaline was injected, the effect of the latter, it will be seen, being still

definitely less than that of 20 γ before ergometrine at A. It will be seen, further, that the pressor effect is reduced in height more than in duration. The effect is not dissimilar to that which might be produced by a small fraction of a mg of ergotoxine, but it cannot be regarded as specific with a dosage of 25 mg It is fairly rapidly evanescent. The cat was left for an hour between C and D, and it will be seen that the effect of 20 γ of adrenaline, given at D, had been completely restored. 7 5 mg of ergotoxine were then injected (2 mg per kg) and, after the usual large pressor effect, pro-

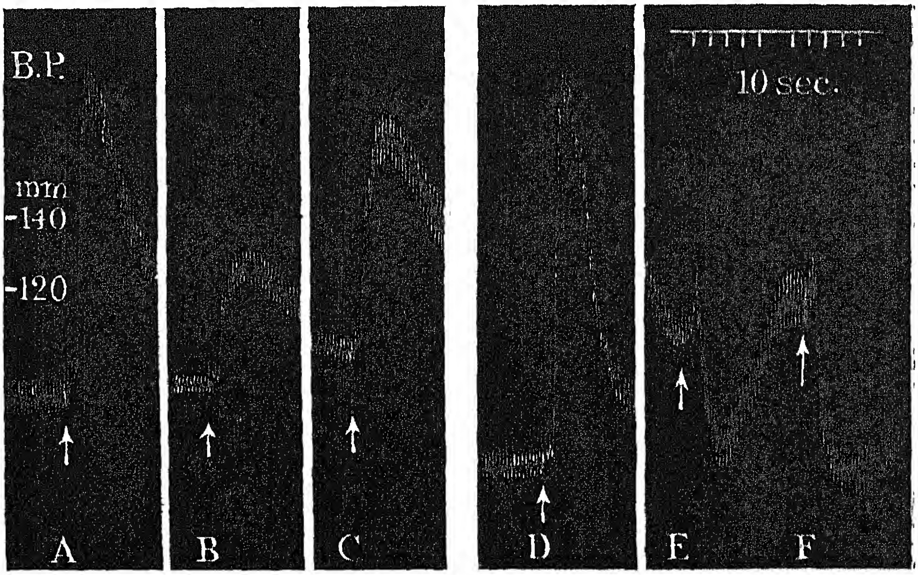


FIG 2—From same experiment as fig 1 A, 20 γ adrenaline. Between A and B, 25 mg ergometrine (*see* fig. 1). B, 40 γ adrenaline. C, 100 γ adrenaline D (one hour later), 20 γ adrenaline. Between D and E, 7·5 mg ergotoxine. E, 40 γ adrenaline. F, 200 γ adrenaline

duced the typical reversal of the effects of adrenaline seen at E (40 γ) and F (200 γ).

Perfused Limbs—The hind limbs of a cat were perfused from the lower end of the aorta, after exclusion by ligature of the internal iliac vessels and transverse lumbar branches, the outflow being collected from the lower end of the vena cava The Dale-Schuster pump was used, and the Hooker Drinker oxygenator As perfusion fluid we used the haemoglobin solution from ox-corpuscles, as described by Amberson and Höber (1932), with which the vascular reactions were remarkably well maintained. The venous outflow rate was also recorded by Gaddum's outflow recorder,

but as the changes thus shown correspond with those to be deduced from the record of the pump pressure, we have reproduced the latter only. In fig 3, the effect is seen, at A, of injection of 1 γ of adrenaline into the tube leading to the arterial cannula. (The small initial disturbance, in this and other records, represents the effect of puncturing the tube, allowing a little perfusion fluid to flow back into the syringe and then emptying the syringe into the system. The effect due to the adrenaline injected is represented by the main, purely pressor phase) At B, 0 2 mg ergometrine was injected. It will be seen that a small preliminary vasoconstriction quickly gives way to a prolonged relaxation of vascular tone. The remainder of

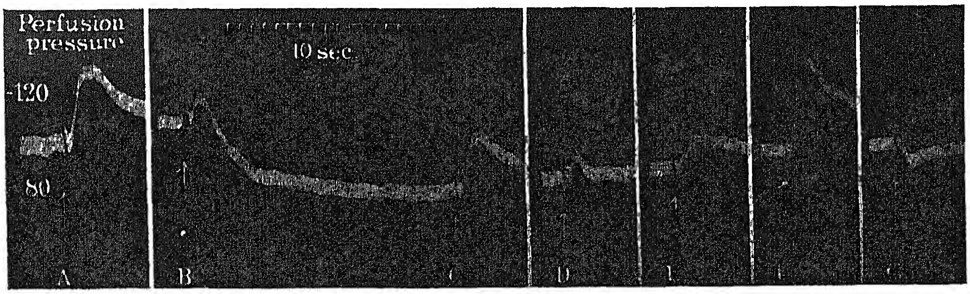


FIG 3—Perfusion of cat's hind limbs Injections into arterial cannula A, 1 γ adrenaline B, 0·2 mg ergometrine. C, 1 γ adrenaline. D, 0·5 mg ergometrine. E, 1 γ adrenaline. F, 5 γ adrenaline Between F and G 0·5 mg ergotoxine G, 5 γ adrenaline.

the record shows the reduction of the vasoconstrictor effect of adrenaline by larger doses of ergometrine, without any trace of reversal, 5 γ at F being about as effective as 1 γ before ergometrine at A 0 5 mg of ergotoxine after F, however, reversed the effect of 5 γ of adrenaline at G, and much larger doses that showed only a vasodilator action

Cat under Anaesthesia—We had expected, from the effect seen in the spinal cat, and from the signs of general sympathetic stimulations produced by an injection of ergometrine into a conscious animal, that it would also show a pressor effect in the anaesthetized cat. It has, on the contrary, invariably produced a well-marked and long fall of arterial pressure in the cat under these conditions. Fig. 4 shows, at A, the effect of injecting 2 mg of ergometrine intravenously into a cat under ether, with artificial respiration. If the animal had been breathing naturally, it is probable that this depressor effect would have been accentuated by a weakening of the respiratory action ; we have seen the pressure partly restored when artificial respiration has been applied at such a stage Fig 4, A, makes

it clear, however, that the depressor effect is, in the main, due to an action independent of the respiratory centre. If a cat, under preliminary ether, is decerebrated anteriorly to an intercollicular section of the mid-brain, and then breathes spontaneously and regularly, ergometrine, given after all the ether has been expired, still produces a fall of arterial pressure associated with respiratory depression; but if the latter is prevented, the animal being kept under an artificial ventilation a little in excess of its respiratory need, ergometrine produces, as in the spinal animal, a pure rise of arterial pressure. Fig. 4, B, shows the pressor effect of 2 mg of ergometrine, after the cat has been decerebrated by intercollicular section and

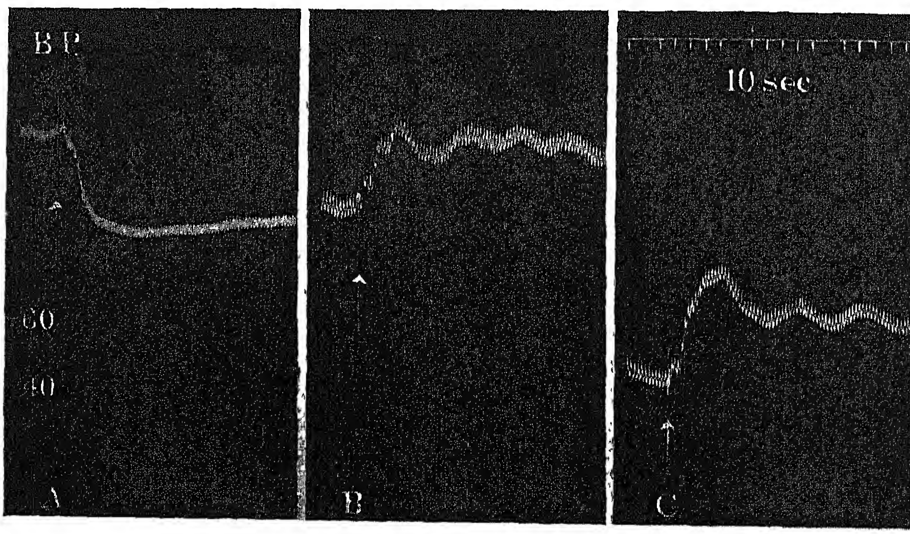


FIG 4—Cat 3·7 kg ether, artificial respiration. Blood pressure. A, B, and C, 2 mg ergometrine intravenously. Between A and B, decerebrated. Between B and C, brain and cord destroyed anterior to second cervical segment.

kept under artificial respiration, and C the effect of the same dose when the animal has been made "spinal" by subsequent section of the spinal cord, at the second cervical segment.

It would appear, from the records on the spinal animal and on the artificially perfused limbs, that the pressor effect of ergometrine is chiefly due to an action on spinal vasomotor centres; and this effect also presumably predominates in the action on the decerebrated animal under artificial respiration. When the fore-brain is intact, even though under the influence of a potent anaesthetic, some centre in it seems to hold a controlling function in maintaining the vasomotor tone, and to be depressed by ergometrine, which causes a fall of arterial pressure under these con-

ditions When this centre has been removed, leaving lower centres in charge of vasomotor tone, ergometrine stimulates these. If the cerebral hemispheres are removed in a cat under ether, and with artificial respiration, a mixture of the two effects is usually seen, ergometrine causing either a slight rise, a slight fall, or both in succession. When it is further remembered that the automatic tone of perfused vessels is also lowered by ergometrine (*see* fig 3, B), it will be clear that the vasomotor effects of the alkaloid are, in any case, the resultants of a complicated antagonism of actions at different levels

Isolated Heart—Davis, Adair, Chen, and Swanson report a depressor effect of " ergotocin " on the perfused heart of the frog We have perfused the heart of a rabbit by the Locke-Langendorff technique and found that ergometrine, injected into the arterial cannula in doses up to 1 0 mg, had no perceptible effect on the rate or amplitude of the ventricular beat

IV—Action on the Uterus

Ergometrine was discovered by its action on the human puerperal uterus, in doses producing no other perceptible effects , and the action on the muscle of the uterus, and particularly of the *post-partum* uterus, is the only specific effect produced in our experiments on animals by small doses of the alkaloid.

We have studied the effect on the uterine muscle of various species and in different conditions in relation to pregnancy Many of the experiments were made on horns or strips of uterus suspended in warm, oxygenated Locke's solution in baths of the usual type The bath volume has in all cases been 15 cc. In other cases the contractions of the uterus have been recorded *in situ* in an animal appropriately anaesthetized, decerebrated, or spinal. A recorder has been used of a type similar to Cushny's myocardiograph, with one fixed rod and one moving on a central bearing. The two rods were attached at their lower ends by sutures to two points placed longitudinally, at a suitable distance, on the horn of the uterus from which the record was to be made The upper end of the fixed rod was made fast in a clamp, and that of the moving rod was attached by a thread passing over pulleys to a weighted writing-lever. The abdominal wall was left widely open, the animal, fixed to a sloping board, being immersed in a large tank of physiological saline solution kept at 38° C, so that the abdominal viscera, including the uterus, were completely covered, the surface of the solution rising to the axillae and only the neck and head projecting above it.

1—UTERUS OF THE GUINEA-PIG

(*a*) *Virgin*—The uterus of the virgin guinea-pig, as shown in Dale and Spiro's comparison of ergotoxine and ergotamine, is extremely sensitive to ergot alkaloids in general. If the two horns of such a uterus are removed and suspended by the usual method in separate baths of Locke's solution, and ergometrine and ergotoxine are added to the respective baths in equal small doses, the responses are very similar, though that to ergometrine will usually be seen to be slightly the more rapid. In fig. 5 a record of such an

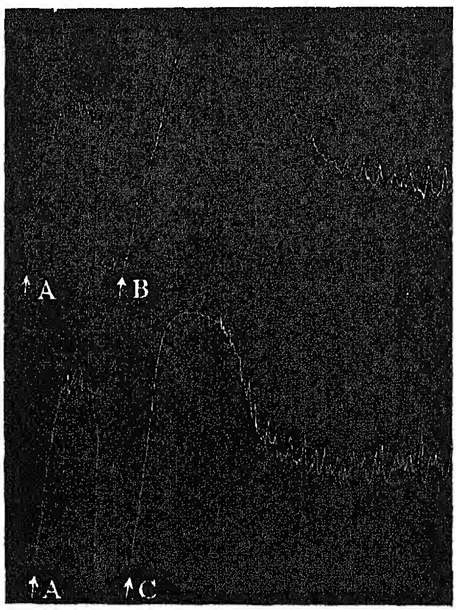


FIG. 5—Symmetrical horns of uterus of virgin guinea-pig. A, histamine, 1 in 10^7. B, ergotoxine ethanesulphonate, 1 in $1 \cdot 5 \times 10^6$. C, ergometrine, 1 in $1 \cdot 5 \times 10^6$.

experiment is shown in which the approximate equality of the activities of the two horns was first ascertained by treating them with equal doses of histamine at A, at B 10 γ of ergotoxine ethanesulphonate were added to the bath containing the horn which gave the upper record, and at C 10 γ of ergometrine were simultaneously added to the other, the concentrations thus produced being in each case $1 : 1 \cdot 5 \times 10^6$. With particularly sensitive preparations reactions can be detected with dilutions down to 1 in 10^9; but there is no definitely preferential sensitiveness to ergometrine over that to ergotoxine, even at such limiting dilutions.

(*b*) *Parous*—The movements of the adult parous, but not pregnant or puerperal, guinea-pig, were recorded from the animal anaesthetized with urethane and immersed in the warm bath. Such a uterus always shows a strong, irregular rhythm, and this and the general tone of the uterine muscle tend to increase as the experiment proceeds 10 γ of ergometrine injected intravenously causes a small increase of tone but has little effect on the already almost maximal rhythm

(*c*) *Puerperal*—In the guinea-pig, as in other species, the most striking effects of ergometrine on the uterus which we have been able to record have been those observed within a few days of parturition The uterus is

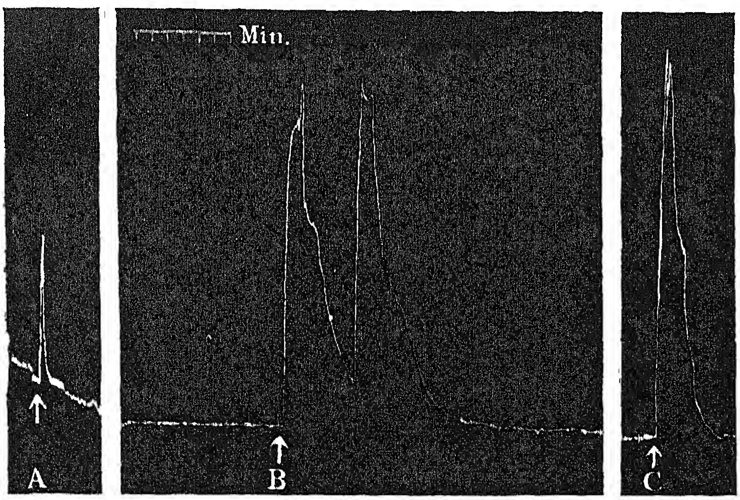


FIG 6 Guinea-pig two days *post-partum* Uterus *in situ* A, Uterus pinched. B, 10 γ ergometrine intravenously. C, Pinch.

then usually large and flaccid, and shows only a minimum of spontaneous rhythm. Even when mechanically stimulated by a firm pinch between finger-ends it gives only a small and evanescent response. Fig 6 is typical of a number of records of this kind which we have obtained from guinea-pigs within two days of parturition After that period the uterus undergoes a rapid involution and returns to the physiological condition above described for the parous non-pregnant organ The record in fig. 6 was taken on a very slowly moving drum. At A, while the uterus was still recovering from slight activity caused by handling during its attachment to the recording system, the horn was firmly pinched and gave only a small and evanescent response. Thirty minutes of complete relaxation and quiescence then elapsed before 10 γ of ergometrine were given intra-

venously at B It will be seen that the response of the uterus by vigorous contractions occurs within a few seconds. These contractions may then continue for hours without any definite intermission; and in such a case a subsequent, even larger, dose of ergometrine has no obvious further effect. In a case such as that shown in fig. 6, there is a long pause after the first group of contractions, and a second dose given during this will produce renewed and then persistent rhythmic activity If, during this pause, mechanical stimulation of the uterus is repeated, as at C in fig. 6, it is found that the sensitiveness of response has been very greatly increased by the

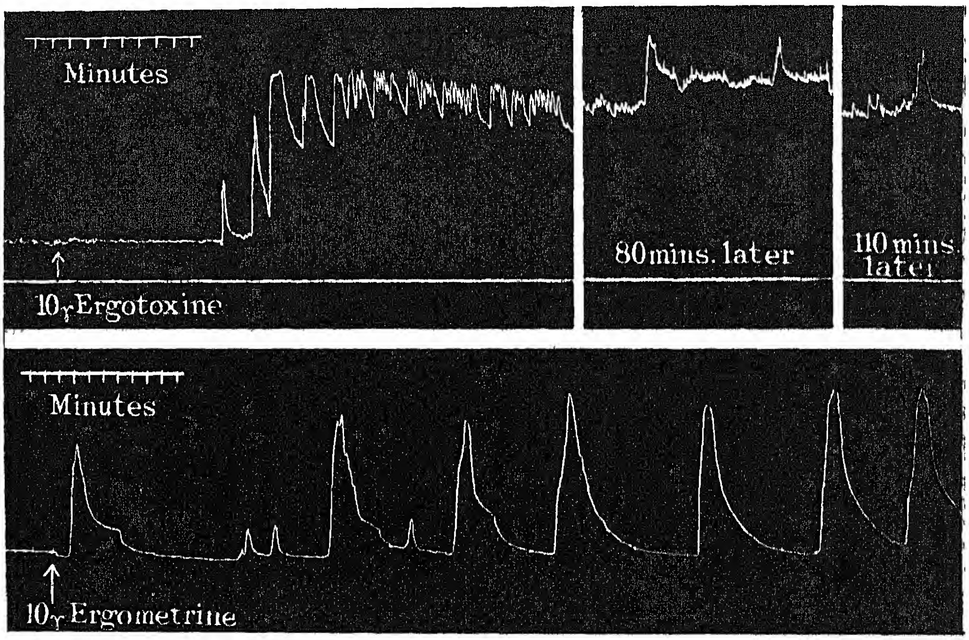


FIG. 7—Records of uteri *in situ* of two guinea-pigs about 12 hours *post-partum*. Upper, 10 γ ergotoxine intravenously Lower, 10 γ ergometrine intravenously.

ergometrine (*cf* fig 6, A and C). In one experiment a strip of uterus, from a guinea-pig killed on the day after parturition, was suspended in a bath as an isolated preparation; but under these conditions it showed such a vigorous and persistent spontaneous rhythm that ergometrine, in concentrations up to 1 in 3 × 10^6, had little perceptible action

Fig 7 reproduces for comparison records from the puerperal uterus *in situ*, made from two guinea-pigs of about the same age and weight, both similarly anaesthetized with urethane The records were made in both cases on the day following parturition, and in both cases the uterus had been quiescent for some time before the beginning of the portion of the

record reproduced The guinea-pig giving the upper tracing received 10 γ of ergotoxine intravenously at the signal It will be seen that there is a latent period of about 10 minutes, and that the uterus then, by a series of increasing contractions, with diminishing relaxation, passes into a condition of strong tonus, with a relatively small rhythm, and that the tonus shows little sign of diminution as the end of the second hour after the injection is approached The guinea-pig which gave the lower tracing received 10 γ of ergometrine by intravenous injection at the signal. With a latency of about one minute, a strong contraction of the uterus occurs, but the relaxation following it is complete This is succeeded by a series of isolated contractions, the amplitude of which tends on the whole to increase, but there is no evidence of any definite tendency to a rise in the level of tone between these contractions

These tracings show the contrast between the actions of the two alkaloids at its strongest Others could be found in which the response to ergotoxine shows more rhythm and that to ergometrine more tone The contrast illustrated is, nevertheless, a genuine one , ergotoxine regularly acts after a long latent period, and tone predominates over rhythm in its effect; ergometrine acts after a short latent period, and rhythm predominates over tone in the response to its action

2—Rabbit

It is probably significant that the uterus of the rabbit, which at all stages of pregnancy gives a motor response to sympathetic impulses and to adrenaline, shows also the most consistent excitatory reaction to ergometrine in all physiological conditions Rothlin has also noted the special responsiveness of the rabbit's uterus to ergobasine, and Davis, Adair, Chen, and Swanson note that the stimulating action of ergotocin in high dilutions on the isolated rabbit's uterus differentiates it from ergotoxine.

In our experience the most appropriate preparation for treatment as an isolated organ is obtained from the thick, coiled horn of the adult multiparous rabbit, by cutting a strip from the junction of such a horn with the broad ligament. In this situation is found a band of plain muscle with predominantly longitudinal disposition, by the contraction of which the rest of the horn is pulled into tight loops and coils. Apart from pregnancy and pseudopregnancy, such a rabbit is practically always in oestrus Such a preparation, when suspended in the warm saline bath, remains for long periods quiescent or shows very small contractions. Fig 8 shows a record made by two strips of this kind, from the same horn, suspended in different baths. Each is first tested with 5γ of adrenaline, giving a concentration of

1 in 3×10^6, and both respond by full contraction At B, the horn producing the upper record receives 20γ of ergometrine (1 in 750,000) and the one producing the lower record 20γ of ergotoxine ethanesulphonate (1 in 900,000 of ergotoxine base) It will be seen that the ergometrine causes a pronounced rhythmic activity without increase of tone, while the ergotoxine has no effect for about 10 minutes, when an isolated short group of contractions occurs, doubtfully attributable to the ergotoxine. After the solution has been changed in both baths, 5γ adrenaline at C causes, in the strip treated with ergometrine, a contraction somewhat greater than that

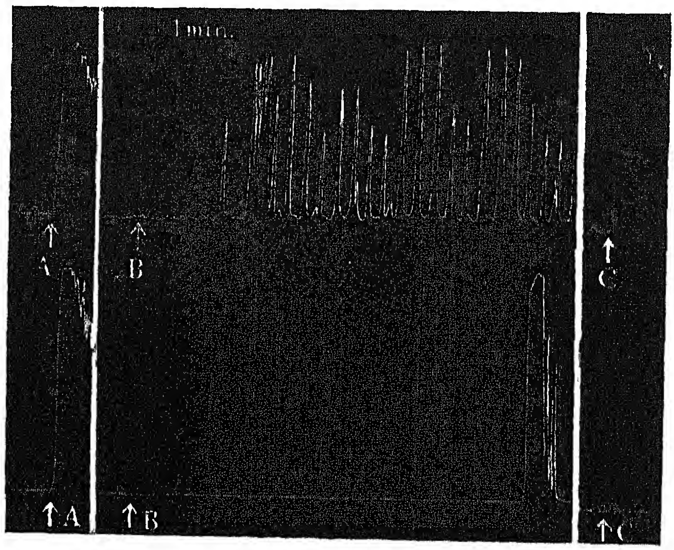


FIG. 8—Oestrous rabbit, strips of uterus from attachment of broad ligament A upper and lower, adrenaline 1 in 3×10^6. B, upper, ergometrine 1 in 750,000 ; lower, ergotoxine, 1 in 900,000. C, upper and lower, adrenaline 1 in 3×10^6. Between B and C the solution was changed.

which it evoked before this treatment, while the strip which received ergotoxine no longer responds at all to this dose of adrenaline

This record is typical of our experience with a number of different kinds of isolated preparations of the rabbit's uterus. In every case the stimulant action of ergometrine on rhythmic activity is greater than that of ergotoxine, and the paralysing action of ergometrine on the motor response to adrenaline, if detectable at all, is much less than that of ergotoxine. In one of the experiments which we carried out according to the method described by Broom and Clark for assay of ergot preparations for ergotoxine, the previously simple augmentor effect of adrenaline was changed by the presence of ergometrine in the bath to a contraction followed by a phase

of inhibition. As soon as the ergometrine was removed by a change of solution, however, the purely augmentor effect of adrenaline was promptly restored; whereas under similar conditions the reversing effect of ergotoxine on the response to adrenaline not only persists after change to fresh solutions but even becomes more complete with successive changes.

Fig. 9 shows a record from the uterus of a non-pregnant rabbit taken *in situ*, with the animal anaesthetized with urethane. The arterial blood pressure is also recorded. It will be seen that 0·5 mg of ergometrine at A causes a prompt contraction, and increased rhythmic activity of the uterus, with a small rise of arterial pressure. Subsequently 0·5 mg of ergotoxine

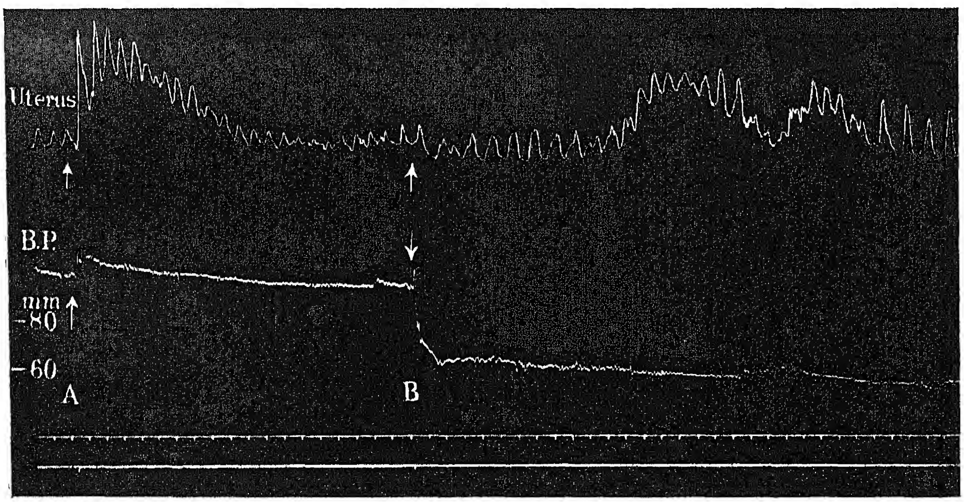


FIG. 9—Rabbit 2·5 kg. Non-pregnant. Record of uterus *in situ* and blood pressure. A, 0·5 mg ergometrine intravenously. B, 0·5 mg ergotoxine intravenously. Time record in minutes.

at B has no immediate stimulating action on the uterus, but causes a prompt and lasting fall of the arterial pressure. An increase of tone and activity of the uterus is seen to occur some 12 minutes after the injection, but it is doubtful whether this can be attributed to the action of ergotoxine.

3—CAT

The non-pregnant uterus of the cat, whether in the virgin or parous condition, and whether treated as an isolated organ or observed *in situ*, has naturally high tone and active rhythm; and though ergometrine increases the tone and activity of such organs, just as ergotoxine and ergotamine had been shown to do (*cf.* Dale and Spiro), there is nothing to differentiate its action on such uteri clearly from that of the earlier known alkaloids. The

same is true of the cat's uterus in the earlier stages of pregnancy. In the cat, as in the guinea-pig, the puerperal uterus gives much the most striking demonstrations of the action of ergometrine Longitudinal bands of muscle, dissected from such an organ and suspended in warm Locke's solution, will often remain for a period of hours in complete relaxation, and without any indication of rhythmic activity The addition of ergometrine to the bath, even in very small doses, then promptly initiates a rhythmic activity, rapidly rising to a maximum, and continuing with great regularity for a subsequent period of hours. Fig 10 gives a record from such a preparation, which had already been suspended for an hour and had remained completely quiescent during that period. At the signal 0·2 γ of ergometrine was added to the bath, giving a dilution of 1 in 75 × 10^6

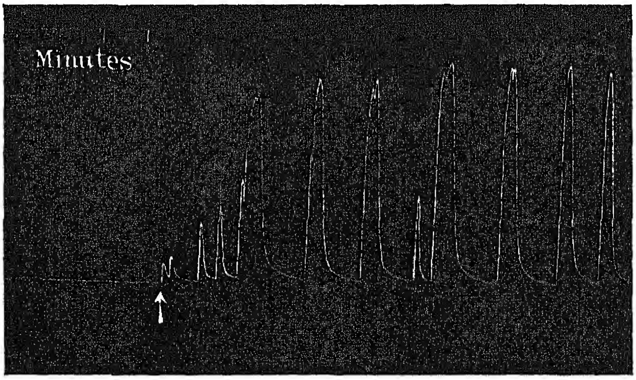


FIG. 10—Cat 12 hours *post-partum*. Isolated strip of uterus. Quiescent for one hour At signal, ergometrine 1 in 75 × 10^6

The puerperal uterus of the cat also furnishes very striking records *in situ*. Fig 11 shows records of carotid pressure and of contractions of a horn of the puerperal uterus in a spinal cat. At A, the recording horn of the uterus was firmly pinched, and gave only a weak and brief response At B 10 γ of adrenaline caused the usual pressor effect, but only a small and brief contraction of the uterus At C, 0 25 mg of ergometrine was injected intravenously The effect on the arterial pressure is not large, though it persists for some time, the uterus, on the other hand, gives a practically immediate and very striking response, beginning a series of powerful contractions, with a slowly rising level of persistent tone. At D, an hour after the injection, the tone and the powerful rhythm are still in evidence This increase of tone is not an invariable feature of the reaction of such a uterus to ergometrine. Fig 12, for example, illustrates an experiment on a decerebrated puerperal cat, with natural respiration.

The uterus had in this case remained completely quiescent for over an hour before the section of the record shown in the figure Early in that period a dose of 0 5 mg of ergometrine had been introduced into the stomach of the cat by a catheter, and had produced no perceptible effect of any kind At A the response of the uterus to a firm pinch was tested The resulting contraction was a little greater in amplitude than one which was recorded before the ergometrine was introduced into the stomach, but the difference was not greater than could be explained by a natural increase of sensitiveness with long exposure in the bath, or by a difference

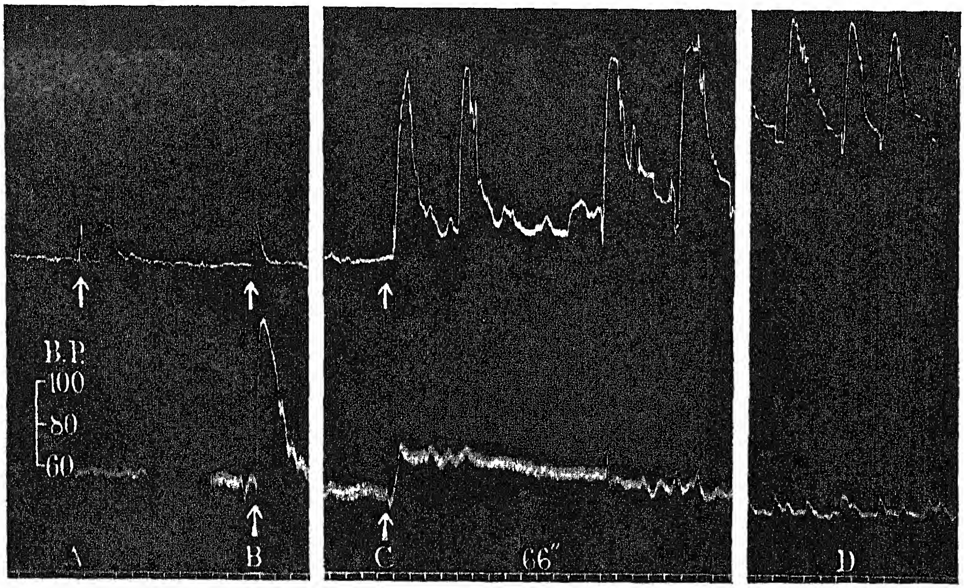


Fig. 11—Spinal Cat. 3 6 kg 2 days *post-partum* Record of uterus *in situ* and blood pressure. A, uterus pinched B, 10 γ adrenaline. C, 0 25 mg ergometrine, i v. D, one hour later N B.—For 66″ *read* 60″

in intensity between the crude mechanical stimuli applied The effect was in any case small and evanescent, and left the uterus apparently as inert as it had been before. At B, 0·5 mg of ergometrine was given intravenously, which caused the immediate onset of a series of maximal contractions of the uterus, with practically complete relaxation in the intervals At C, 35 minutes later, the series still continued with undiminished vigour but also without any persistent tone It will be seen that, at B, the blood pressure begins to fall immediately after the injection of ergometrine This fall was associated with a rapid decline of the natural respiration, and was arrested by application of artificial ventilation at AR.

In one case we were able to obtain a cat during parturition Three kittens from the left horn of the uterus had already been born and the head of the leading one of two kittens in the right horn was descending into the pelvis The brain of the cat was destroyed under ether, and arrangements were made to record the contractions of the empty left horn of the uterus, leaving the right horn free to continue its expulsive activity It was observed that the contractions of the empty horn were synchronous with those which, in the still pregnant horn, continued the parturient effort

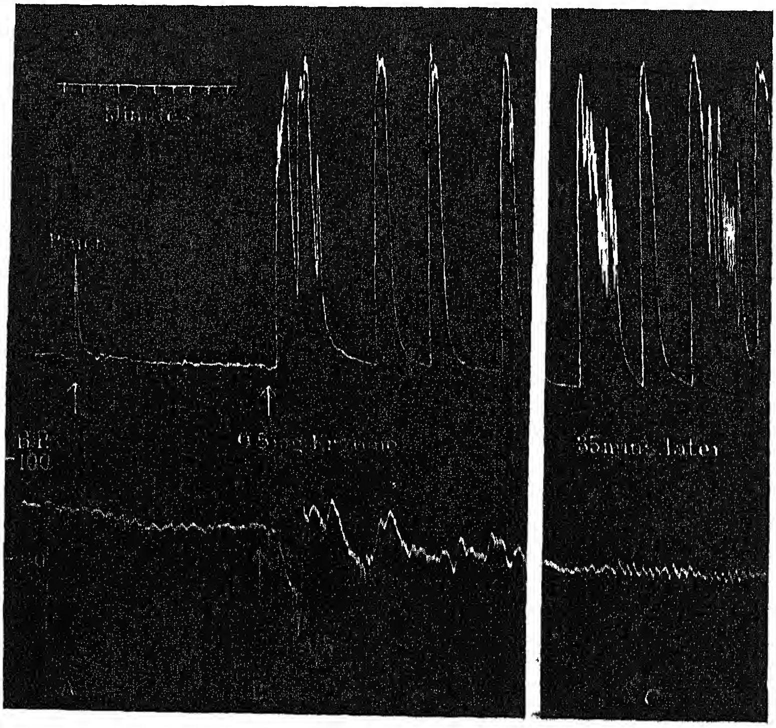


FIG 12—Decerebrate Cat. 3 kg 24 hours *post-partum* Record of uterus *in situ* and blood pressure. A, uterus pinched B, 0·5 ergometrine, i.v. C, 35 minutes later

The spontaneous activity of both was so great that we did not expect to see much effect from ergometrine The intravenous injection of 20γ of that alkaloid did, however, produce a definite increase in the frequency of the contractions and some rise in the general level of tone This adjuvant effect lasted for about an hour We then tried the effect of a larger dose, injecting 150 γ, but this caused only a renewed increase in the rate and amplitude of rhythm of about the same dimensions as 20 γ had previously produced. When the apparatus was detached, and the hind limbs freed

from constraint, it could be seen that each phase of contraction caused movement of the foetus towards the pelvis, but presumably the co-operation of the voluntary muscles of the abdominal wall would be required to bring about birth

4—Other Species

The uterus of the hamster was examined only in the non-pregnant condition and only as an isolated organ A horn of a hamster's uterus, suspended in warm Locke's solution, shows a small regular rhythm arising from a constant base-line Its activity is inhibited by adrenaline, and fairly sensitive to its action, being promptly relaxed by a concentration of 1 in $7 \cdot 5 \times 10^6$. On the other hand, it is relatively insensitive to histamine, though not so insensitive as the uterus of the rat Histamine in a concentration of 1 in 30,000 caused contraction of the hamster's uterus on first application; but, when reapplied after thorough washing of the preparation, it became progressively less sensitive with subsequent applications Ergometrine then caused a pronounced increase of tone, followed by relaxation and greatly augmented rhythm, when applied in a concentration of ·1 in 75,000 Ergotoxine acted in a similar dilution, but produced a persistent tonus, little broken by rhythm

We made a few experiments also on isolated horns of the uterus of the ferret and the rat, but in neither case obtained any striking or specific effect of ergometrine, the organs showing in both species a very vigorous spontaneous rhythm

V—Actions on other Isolated Tissues

Seminal Vesicle

Rothlin (1925) has recommended the seminal vesicle of the guinea-pig as a useful test object for the quantitative determination of ergotamine, in terms of its paralysing action on the motor effect of adrenaline. We also observed that this organ, suspended in the saline bath, reacted by contraction and rhythm to the addition of adrenaline in a concentration of 1 in 4×10^6. The effect was easily annulled by ergotoxine, the application of which in a concentration of 1 in 4×10^6 reduced the effect of adrenaline by 75%, while further treatment with ergotoxine in a strength of 1 in $1 \cdot 5 \times 10^6$ almost completed the annulment of the adrenaline action On the control vesicle, tested at the same time, and with the weight adjusted so that it gave a contraction of equal amplitude to adrenaline in the same concentration, ergometrine had no effect at all, even when applied in a

concentration of 1 in 3×10^3; the vesicle thus treated still gave an undiminished response to adrenaline This provided, perhaps, the sharpest and clearest contrast between a potent paralysing effect of ergotoxine on a motor action of adrenaline and complete absence of such action by ergometrine in much higher concentration.

SMALL INTESTINE

(*a*) *Rabbit*—On a loop of rabbit's jejunum suspended in Tyrode's solution and showing its normal regular rhythm ergometrine, in a concentration of 1 in 10^5, produces a weak but quite definite inhibitor effect Various effects on other organs, and particularly the readier response to ergometrine of the rabbit's non-pregnant uterus than that of other animals, had suggested that the action of ergometrine had a sympathomimetic component; and this inhibitor effect on the rabbit's intestine seemed to conform to that type. It has been described by Davis, Adair, Chen, and Swanson, who found that, in accordance with the view that it is sympathomimetic in nature, it is abolished by ergotoxine We also had observed that ergotoxine in a concentration of 1 in 10^5, though it produces no perceptible direct effect on the intestinal rhythm, entirely annuls the inhibitor action of ergometrine subsequently applied Ergotoxine in this concentration also weakens the inhibitor action of adrenaline on the rabbit's intestine

(*b*) *Guinea-Pig*—In contrast to its action on that of the rabbit, ergometrine produces a tonic contraction of a loop of the guinea-pig's jejunum, in concentrations as low as 1 in 10^6 The effect rapidly subsides on changing the solution, and can apparently be repeated indefinitely. There is no proper ground for a suggestion that this stimulant effect on the guinea-pig's intestine is sympathomimetic, the action of adrenaline being purely inhibitory, nevertheless this action of ergometrine is readily annulled by ergotoxine, which again exhibits no significant direct effect of its own on the activity of the intestinal muscle It should be mentioned, further, that this paralytic effect of ergotoxine, unlike its very persistent annulment of sympathetic motor effects, appears to be removed without difficulty by changing the fluid in the bath; after clean Tyrode's solution has been substituted and replaced a few times the intestinal muscle regains its motor response to ergometrine.

An isolated loop of the small intestine of a cat showed no response to ergometrine or to ergotoxine applied in concentrations as high as 1 in 3×10^4.

VI—Discussion

The action of ergometrine, as described and analysed in the foregoing sections, gives indications at different points of a peripheral action of the sympathomimetic type This, however, is weak in itself, and is overlaid and obscured by actions of other types, and in particular, as seen in the unanaesthetized animal, by an excitatory effect on mid-brain centres, to which the syndrone of general sympathetic stimulation is chiefly due, and, especially in the cat, on higher brain centres as well. In these respects its action is linked, on the one hand, to that of a substance like β-tetrahydronaphthylamine, and, on the other hand, to those of the more complex ergot alkaloids hitherto known From these latter it is differentiated in action by an almost complete absence of the specific paralysing effect on augmentor sympathetic actions and by, at most, a much weaker activity in the production of gangrene

While with relatively large dosage ergometrine thus shows such associations and contrasts of activity with other substances, the only specific and characteristic effect which can be attributed to it, when it is administered in a dosage of the order of that which has hitherto been clinically applied, is that action on the uterus for which it has been clinically used and which led to its discovery It is only in the puerperal uterus of certain species that we find the association of quiescence with potential responsiveness, which appears to be necessary for the clear demonstration of this specific effect. Even in the rabbit, with the motor response of its uterus to sympathomimetic substances persisting through all functional phases. it is only when the spontaneous activity of the uterus is slight that the action of ergometrine on it can be clearly shown When a uterus, like that of the oestrous guinea-pig or the puerperal rat, has already a vigorous spontaneous rhythm, no dose of ergometrine will greatly enhance it Its typical action is to evoke the immediate onset of a strong rhythm, where this was absent. Its action in this direction differs from that of ergotoxine, and presumably of the other complex ergot alkaloids, even when both are given intraveneously or applied to the isolated organ, in the absence of any significant delay in onset and in the predominantly rhythmic nature of the activity evoked; ergotoxine, by contrast, usually acting with a delay of some minutes and then producing a response in which tone predominates over rhythm. There is one other difference, of primary practical interest, between the two kinds of alkaloid which we have not been able to demonstrate to our satisfaction in our experiments on the uterus of different animals, namely, the relative rapidity with which ergometrine is absorbed and comes into action when given by the mouth By giving large doses

by mouth to unanaesthetized animals, such as the cock or the cat, and watching the onset of toxic effects, we have been able to obtain evidence, indeed, of the much greater rapidity of absorption of ergometrine than of ergotoxine In experiments on the uterus, on the other hand, whether the animals were under chemical anaesthesia, or were decerebrate or spinal, we observed no regularity of response to ergometrine given by the mouth in doses which, when injected intravenously, produced an immediate and striking effect When once ergometrine had been isolated, and its action on the uterus with oral administration was known from clinical experience, it doubtless became possible, on occasion, to demonstrate this action on an anaesthetized animal. We are convinced, however, that in dealing with impure extracts the action with oral administration to anaesthetized animals would have been too irregular, and the action with parenteral injection, or application to isolated tissues, too much complicated by the presence of other ergot alkaloids and of histamine for any certain recognition of the presence of an additional activity due to a substance with the properties of ergometrine. For that purpose it was necessary to observe. and to record objectively, the effect on the uterus of the human patient, willing to co-operate and able to lie still, for hours if necessary, without anaesthesia. It was not, in fact, until Moir made experiments of this kind that the activity due to this most easily isolated of all ergot alkaloids was recognized; and it was the continuous application of this clinical control, at each stage of Dudley's chemical work, which made the isolation possible. When the nature of the problem is realized, we do not think that it is difficult to understand that this active principle of ergot, probably the most practically important of all, should for so many years have escaped detection by the methods of conventional pharmacology

VII—Summary

The paper describes the pharmacological actions of ergometrine, the alkaloid recently isolated from ergot by Dudley and Moir (1935).

1—Ergometrine produces the central excitation, with general sympathetic stimulation, previously described as an initial phase of the action of the more complex alkaloids of the ergotoxine group It has, however, no more than a trace of the specific paralysing action on motor sympathetic effects, characteristic of these other alkaloids It causes cyanosis of the cock's comb, but the effect is evanescent and has, in our experience, not led to gangrene in a course of injections which, with ergotoxine, produced an extensive gangrene of the comb. Like ergotoxine again, it causes a rise of body temperature when given in toxic doses. It is, in general, less

toxic than ergotoxine, and is much more readily absorbed with oral administration.

2—The actions of ergometrine on the arterial pressure vary with the conditions of anaesthesia, or of integrity of the brain. In the spinal cat it has a pressor action, but far less than that of the alkaloids of the ergotoxine group

3—The most characteristic action of ergometrine, and the only one produced by small doses, is the initiation of a long persistent rhythm of powerful contractions in a uterus normally quiescent, as in the early puerperium.

4—On several organs, apart from the centrally excited sympathetic stimulation, ergometrine appears to have a peripheral action of sympathomimetic type This, however, is much complicated by other types of action, and does not account for its specific action on the uterus

References

Amberson, W. R, and Hober, R (1932) 'J cell comp Physiol,' vol 2, 201.

Barger, G, and Dale, H H (1907) 'Biochem J,' vol 2, 240.

Barger, G, and Dale, H H (1909) 'Arch exp Path Pharmak,' vol. 61, p 113.

Dale, H H, and Spiro, K (1922) 'Arch. exp Path Pharmak,' vol 95, p 337.

Davis, M E, Adair, F L., Chen, K K, and Swanson, E E (1935). 'J Pharmacol.,' vol 54, p 398

Dudley, H W, and Moir, J C (1935) 'Brit Med J,' vol 1, p. 520

Jacobs, W. A., and Craig, L C. (1935) 'Science,' vol 82, p 16

Kreitmair, H (1934). 'Arch exp Path Pharmak,' vol. 176, p. 171.

Moir, J C (1932). 'Brit Med J,' vol. 1, p 1119

Rössler, R, and Unna, K (1935) 'Arch exp Path Pharmak,' vol 179, p 115.

Rothlin, E (1925). 'Klin Wschr,' vol. 4, p 1437.

Rothlin, E. (1935) 'C R. Soc Biol., Paris,' vol 119, p 1302.

Thompson, M. R (1935). 'J Amer. pharm. Ass,' vol. 24, p. 185

547 945 I

Ergometrine

By HAROLD WARD DUDLEY, F R S.

(From the National Institute for Medical Research, Hampstead, N.W 3)

(*Received August* 15, 1935)

[PLATE 27]

INTRODUCTION

In consequence of the observation of Moir (1932) that ergot extracts, administered orally, provoked prompt and vigorous contractions of the human puerperal uterus which could not be attributed to any of the known constituents of ergot, Dudley and Moir undertook a systematic search for the responsible substance, and eventually succeeded in isolating it (Dudley and Moir, 1935, Dudley, 1935, *a*).

It proved to be a hitherto unknown alkaloid related to, but evidently simpler than, the six or seven alkaloids which had already been isolated from ergot, and was given the name *ergometrine.*

Its essential nature and characteristics having been determined with the small quantity first obtained, it proved easy to devise a method of preparation much simpler than that which led to the isolation of the original specimen This method has already been published (Dudley, 1935, *b*), and renders ergometrine by far the most readily accessible alkaloid of ergot

A precise chemical description of ergometrine, promised in our first communication, is the object of this paper.

PROPERTIES OF THE FREE ALKALOID

Ergometrine is a white crystalline substance, is freely soluble in the simple alcohols, ethyl acetate, and acetone and methyl ethyl ketone, sparingly soluble in dichloroethylene and benzene, and very sparingly soluble in chloroform, it crystallizes best from benzene (400 parts) as slender white needles or from methyl ethyl ketone (10 parts) as stout prisms, m pt 162–3° (decomp) (*see* Plate 27)

The behaviour of ergometrine on crystallization from ethyl acetate is worthy of note

A solution of 0 5 gm of the alkaloid in approximately 20 cc of ethyl acetate, kept at −4° deposited 0 22 gm of white lustrous thin plates, melting at 160–1°, which proved to be ergometrine with no solvent of

crystallization The mother liquor on concentration in a vacuum to about 10 cc became filled with needles These were dissolved by warming, and the solution, cooled to room-temperature, deposited 0 14 gm of diamond-shaped plates, which melted with effervescence but no darkening at 130–132° This low-melting substance was found by analysis to be ergometrine combined with ethyl acetate of crystallization in the proportion of 0 5 mol per mol of ergometrine Recrystallization from methyl ethyl ketone converts it to the usual form obtained from this solvent, melting at 162–3°

The ethyl acetate of crystallization is much more firmly combined with ergometrine than are either benzene or methyl ethyl ketone with the alkaloid crystallized from these solvents Exposure to a temperature of 100° in a high vacuum removes the solvent of crystallization from ergometrine crystallized from benzene or methyl ethyl ketone, but leaves the ethyl acetate containing crystals intact The low melting-point of the ergometrine-ethyl acetate crystals as compared with that of the alkaloid-benzene or -methyl ethyl ketone crystals is evidently due to the stability of the combination between the alkaloid and solvent of crystallization in the former Another interesting difference between the ethyl acetate-containing crystals and those containing benzene or methyl ethyl ketone was observed.

The latter, even when kept in darkness, are readily oxidized on exposure to air, becoming brown in colour, the former are much less susceptible to this attack The superficial browning of all three forms is much accelerated by exposure to light Ergometrine is moderately soluble in water yielding a blue-fluorescent, dextrorotary solution with a reaction alkaline to litmus, which, on account of oxidation, turns brown if exposed to the air

Ergometrine gives Keller's (ferric chloride), Van Urk's (*p*-dimethyl-aminobenzaldehyde), and the glyoxylic acid reactions, indicative of the presence of an indole nucleus and common to the other known ergot alkaloids. It is a mono-acid base, yielding a variety of crystalline salts

The Empirical Formula of Ergometrine · $C_{19}H_{23}O_2N_3$

The analysis of the free alkaloid is fraught with difficulties, due to its instability, its hygroscopic nature, and the risk of incomplete removal of solvent of crystallization. Results selected from twelve analyses made, fit the formula given above perfectly, but it could not be derived with certainty from the whole series until a determination of equivalent weight had been made by analysis of the picrate

The above formula was then indicated and confirmed by analyses of the hydrochloride, hydrobromide, and oxalate, stable salts containing (as prepared) no solvent of crystallization, and therefore much more suitable for analysis than is the free alkaloid.

Analyses

Ergometrine (from ethyl acetate: solvent-free)

Found: Loss of wt. at 90° in high vacuum, 1·1%.
C 70·0, H 6·96, N 12·91%.
Calc. for $C_{19}H_{23}O_2N_3$: C 70·15, H 7·08, N 12·92%

Ergometrine (containing ethyl acetate of crystallization: low melting).

Found: Loss of wt. at 100° in high vacuum 0·39%.
C 68·21, H 7·40, N 11·80%.
Calc. for $C_{19}H_{23}O_2N_3$, 0·5 $C_4H_8O_2$: C 68·30, H 7·32, N 11·38%.

Ergometrine picrate (red form, *see* p. 482).

Found: No loss of wt. at 100° in high vacuum.
C 54·48, H 4·32, picric acid (by nitron) 41·8 and 41·5%, whence equiv. wt. of base 319 and 323
Calc. for $C_{19}H_{23}O_2N_3$, $C_6H_2O_7N_3$: C 54·25, H 4·52%
Mol. wt. of base 325.

Ergometrine picrate (yellow form, *see* p. 482).

Found: Loss of wt. at 100° in high vacuum, 3·23%.
Calc. for $C_{19}H_{23}O_2N_3$, $C_6H_2O_7N_3$, H_2O: 3·15%

Ergometrine hydrochloride.

Found: No loss of wt. at 100° in high vacuum.
C 63·1, H 6·81, N 11·29, Cl 9·54%.
Calc. for $C_{19}H_{23}O_2N_3$, HCl: C 63·08, H 6·64, N 11·62, Cl 9·82%.

Ergometrine hydrobromide.

Found: No loss of wt. at 100° in high vacuum
C 56·24, H 6·19, N 10·71, Br 19·76%
Calc. for $C_{19}H_{23}O_2N_3$, HBr: C 56·15, H 5·91, N 10·35, Br 19·71%.

Ergometrine oxalate

Found: No loss of wt. at 100° in high vacuum.
C 60·58, H 5·98, N 10·18%.
Calc. for $C_{19}H_{23}O_2N_3$, $C_2H_2O_4$: C 60·73, H 6·02, N 10·12%.

Optical Activity of Ergometrine and its Salts

Rotation of Ergometrine in Chloroform

The original specimen of ergometrine displayed a negative rotation in chloroform, an observation which served to differentiate it at that stage from sensibamine, an ergot alkaloid about which only meagre details were available in a patent specification (Kereszty and Wolf, 1933) Although the very slight solubility of ergometrine in chloroform precludes a satisfactory determination of specific rotation in this solvent, it was of interest to check the observation on highly purified material Accordingly 0 01 gm of ergometrine (m pt 162–3°, containing 10 33% of methyl ethyl ketone of crystallization) was dissolved in 10 cc of chloroform by heating On cooling to 24° some ergometrine crystallized out of the solution The observed rotation of the saturated solution in a 4 dm tube using light of λ 5461 was −0 145°

Specific Rotation of Ergometrine in Ethyl Alcohol

0·1991 gm of ergometrine containing methyl ethyl ketone of crystallization, m. pt 162–3° (= 0·1785 gm of solvent-free alkaloid) dissolved in 10 cc of ethyl alcohol had $[\alpha]_{5461}^{20} = +59\cdot2°$, $[\alpha]_D^{20} = +40\cdot25°$.

Specific Rotation of Ergometrine Hydrochloride in Water

$C = 0\cdot8734$ $[\alpha]_{5461}^{25} = +87\ 6°$; $[\alpha]_D^{25} = +63°$.

Specific Rotation of Ergometrine Oxalate in Water

$C = 0\ 5960 \cdot [\alpha]_{5461}^{23} = +76\ 2°$, $[\alpha]_D = +55\cdot4°$.

Specific Rotation of the Ergometrine Ion in Water

Calc from the values for the hydrochloride:

$[\alpha]_{5461}^{25} = +97\cdot5°$; $[\alpha]_D^{25} = +70\ 1°$

Calc from the values for the oxalate:

$[\alpha]_{5461}^{23} = +97\ 3°$; $[\alpha]_D^{23} = +70\ 7°$

Salts of Ergometrine

Ergometrine Hydrochloride

0 1 gm of ergometrine (recrystallized from methyl ethyl ketone) was dissolved in 15 cc of acetone The addition of 0·34 cc *N*–HCl (aqueous) to this solution caused the rapid crystallization of the hydrochloride, 0·08 gm of which was obtained. It crystallizes in needles and may be recrystallized from ethyl alcohol: m. pt (with decomposition) 245–6°.

Ergometrine hydrobromide was prepared similarly to the hydrochloride, and crystallized more slowly than the latter in needles (*see* Plate 27) M pt (with decomposition) 236–7° It is less soluble in water than is the hydrochloride Both salts crystallize well from water.

Ergometrine oxalate was prepared by dissolving 0·2 gm of ergometrine (recrystallized from methyl ethyl ketone) in 15 cc of ethyl alcohol and adding 0 1 gm of oxalic acid dissolved in 8 cc of ethyl alcohol. The salt separated in very fine needles (0·2 gm) and was recrystallized from 96% alcohol (*see* Plate 27) It melts with decomposition at 193°.

Ergometrine Picrate

The behaviour of ergometrine with picric acid is very characteristic If ergometrine is dissolved in warm aqueous picric acid and the solution is allowed to cool, or if an aqueous solution of the hydrochloride is treated with a solution of sodium picrate, two very different types of crystals are usually obtained together, one appearing as golden yellow needles and the other as short ruby-red columns. The yellow crystals are more readily soluble in hot water than the red, and the latter can be separated by warming the solution until all the yellow material is dissolved and filtering. The filtrate on cooling, however, still deposits a mixture of the two types of crystals, and this also happens if the homogeneous red material is recrystallized from hot water. Methods were devised for obtaining each type of crystals free from the other, and they both proved to be ergometrine picrate, the yellow needles being a hydrated form and the red columns the anhydrous salt (*see* Plate 27) They are interconvertible by the methods given below. Ergometrine picrate was prepared by adding 1 gm of ergometrine (from benzene) dissolved in 100 cc of water containing 2·2 cc of N–H_2SO_4 to a solution of 2 gm of picric acid in 100 cc of hot water. 1 6 gm of ergometrine picrate, mainly in the yellow form, were obtained.

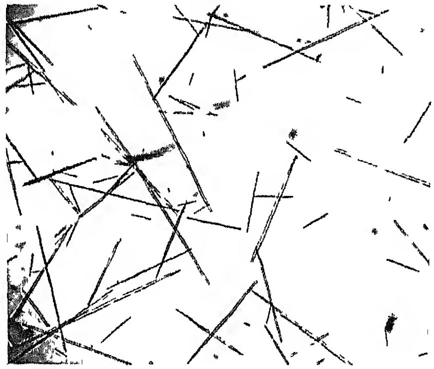
Preparation of Yellow Ergometrine Picrate

0 3 gm of the picrate was dissolved in about 20 cc of 50% acetone. Water was added until a permanent turbidity was produced The liquid was warmed on a water-bath until it was clear, and then allowed to cool. 0 26 gm of yellow fine needles was obtained, m pt (with decomposition) 148°

Preparation of Red Ergometrine Picrate

0·3 gm of ergometrine picrate was dissolved in 14 cc of hot 90% alcohol. Ruby-red prismatic columns were deposited on cooling If, as sometimes

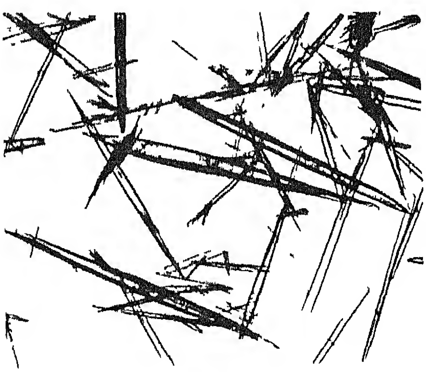
Ergometrine (from methyl-ethyl ketone)



Ergometrine (from benzene)

Ergometrine picrate (red form)

Ergometrine picrate (yellow form)



Ergometrine hydrobromide

Ergometrine oxalate

(All at same magnification)

happens, a small amount of the yellow form appears, the liquid should be warmed on a water-bath until this is redissolved, and again allowed to cool The perfectly homogeneous red form will eventually be obtained 0 22 gm was recovered m pt. (sudden decomposition) 188–9°.

The Relationship between Ergometrine, Ergotocin (Kharasch and Legault), and Ergobasine (Stoll and Burckhardt)

Some confusion has arisen in the literature on account of statements made by Kharasch and Legault and by Stoll and Burckhardt which create the impression that the alkaloids which they have recently isolated from ergot are different from ergometrine

Both these groups of workers lay emphasis which cannot be scientifically justified on differences between data given in our original communication —a preliminary account in a clinical journal—and those which they have subsequently obtained.

For instance, Kharasch and Legault lay great stress on the difference between the decomposition point of ergometrine (152°) given in our first account, and that found by them subsequently for highly purified material (162°). It is only necessary to recall that in their first description of "ergotocin" Kharasch and Legault (1935, *a*) gave the decomposition point as 155°.

A comparable example may be taken from the paper of Stoll and Burckhardt (1935) They drew special attention to the fact that, whilst a solution of "ergobasine" in water exhibits a positive rotation, we reported a negative rotation for a solution of ergometrine in chloroform. From this contrast they conclude that ergometrine and ergobasine are chemically different substances.

It is a matter of common knowledge that such a conclusion is inadmissible, because one and the same substance may display a positive or negative rotation according to the solvent chosen for the determination. An example of this phenomenon has occurred in Stoll's own work on another ergot alkaloid, ergotamine, for which he records a negative rotation in chloroform and a positive one in alcohol.

It has been confirmed that ergometrine has in fact a negative rotation in chloroform, whilst in water it is dextrorotary. In alcohol also it exhibits a positive rotation, behaving as regards optical activity in chloroform and alcohol like Stoll's ergotamine.

Further examination of arguments which cannot serve any scientific purpose is unnecessary Actually, although its method of preparation has not yet been disclosed, and descriptions of its salts are meagre, the

substance now called "ergotocin" is in all probability identical with ergometrine, notwithstanding the formula $C_{21}H_{27}O_3N_3$ recently attributed to it by Kharasch and Legault (1935, *b*), which will probably have to be abandoned in favour of that established for ergometrine in the present communication This latter agrees with Stoll and Burckhardt's results for "ergobasine," a perusal of which leaves no room for doubt concerning the identity of that substance with ergometrine.

References

Dudley (1935, *a*) 'Brit Med J,' vol. 1, p 798

—— (1935, *b*) 'Pharm J,' vol 80, p 709.

Dudley and Moir (1935) 'Brit Med J,' vol 1, p 520

Kereszty and Wolf (1933) 'Chem Abs,' vol. 27, p 3779

Kharasch and Legault (1935, *a*) 'J. Amer Chem Soc,' vol 57, p 956.

—— (1935, *b*) 'J Amer Chem Soc.,' vol 57, p 1140

Moir (1932) 'Brit Med J,' vol 1, p 1119

Stoll and Burckhardt (1935) 'Bull Sci pharmacol,' vol 42, p 257; *see also* 'C R Acad Sci,' Paris, vol 200, p 1680

612 018 2

Vestigial Structures Observed in Male Mice After Prolonged Treatment with Oestrogenic Compounds

By HAROLD BURROWS, Research Institute, The Cancer Hospital (Free), London

(*Communicated by E L. Kennaway, F R S—Received May* 14, 1935)

[PLATES 28 AND 29]

Three hundred and seventy-three male mice were subjected to various oestrogenic compounds for periods of 50 days or more, in order to learn whether these compounds might affect the incidence of cancer In each experiment, one drop of a solution (0·3 to 0 01%) of oestrogenic compound in benzene or alcohol was applied twice a week by means of a small paint brush to the skin of the interscapular region This treatment causes pronounced changes in the coagulating glands which become converted into simple sacs lined by a squamous keratinizing epithelium, distended with keratinous debris, and frequently also with pus (Burrows and Kennaway, 1934 , Burrows, 1935) At a later date the seminal vesicles undergo a similar transformation. These changes are shown in the appended illustrations. In twenty-two of the mice treated in this way, abnormal structures have been found either (1) just dorsal to the prostatic urethra, or (2) in the neighbourhood of the epididymis These structures have consisted of single cysts in 20, of a collection of tubules in one, and of a single cyst and a tubular structure in another In all instances the structures have been lined with squamous keratinizing epithelium.

1 *Cysts Dorsal to the Prostatic Urethra*—These cysts, of which there are 17 examples, have not been seen to communicate with the urethra It is possible that in some instances they do so. Complete serial sections have been made in two cases only, and in these the cavity of the cyst was not in communication with the urethra or any neighbouring organ In other examples many serial sections have been made without revealing any communication between the cavity of the cyst and adjacent structures. From their situation and relationships it seems possible that these keratinized cysts may represent vestiges of the caudal end of Muller's duct brought into prominence through the metaplastic action of the oestrogenic compounds administered The cysts lie dorsally to the urethra, where a uterus masculinus might be expected to lie The vasa deferentia pass downward in their lateral walls, figs. 1, 2, and 4, Plate 28, and then lie close together

near the mid-line between the cyst and the urethra, where they become the ejaculatory ducts, figs 3, 4, and 5, Plates 28 and 29 In some instances the cysts project freely into the abdominal cavity and are seen as glistening domes just dorsal to the bladder, figs. 1, 2, and 3, Plate 28 ; in others the lateral wall of the cyst may be incorporated on each side with those of the coagulating glands, fig 5, Plate 29

2. *Cystic or Tubular Vestiges in the Neighbourhood of the Epididymis*—Five examples have been seen. In three of these, figs 6, 7, and 8, Plates 28 and 29, keratinized single cysts were attached to the vas deferens on the proximal side of the gubernaculum testis, in one instance 3 mm from this structure Complete serial sections through two of these, fig 6, Plate 29, show that the cavity of the cyst is completely closed, its walls being connected with the vas deferens by loose areolar tissue In two cases, fig 8, Plate 29, there was a mass of tubules lined with squamous, keratinized epithelium, attached in one to the vas deferens at a point not exactly ascertained, but apparently close to the tail of the epididymis, and in the other, fig 8, Plate 29, incorporated in the head of the epididymis In the latter case there was, in addition, a single keratinized cyst attached to the vas deferens at its emergence from the tail of the epididymis

It seems probable that these cysts might be regarded as persistent remnants of the cephalic end of Muller's duct, rendered obvious by a metaplasia induced by the oestrogen administered. Their loose attachment to the vas deferens seems to suggest that they are Müllerian and not Wolffian vestiges.

Perhaps additional examples of the two types of vestigial structure would have been discovered if all the mice had been more carefully examined for the special purpose ; but it is certain that they were not present in a large percentage. A systematic search by serial sections of the organs in question in untreated mice would be required to show whether rudiments of the structures described here exist normally. There has been no opportunity to make such a search They have been looked for with the naked eye in a large but unrecorded number of mice, probably one hundred at least, which had not been treated with an oestrogen, and have not been found, although some of these structures are so large that they could not have been overlooked, fig 2, Plate 28. Nor have they been found amongst the relatively few microscopical sections which have been made from the genitalia of such mice.

Summary

Abnormal structures, consisting of cysts or tubules lined with squamous keratinizing epithelium, have been found in male mice after the prolonged

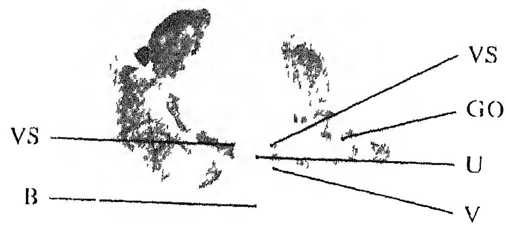


FIG 1

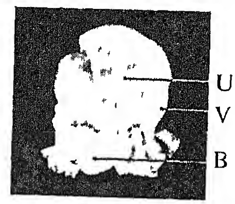


FIG 2

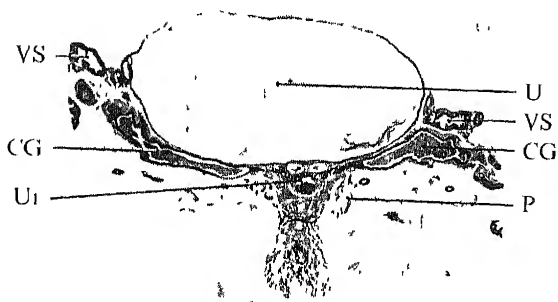


FIG 3

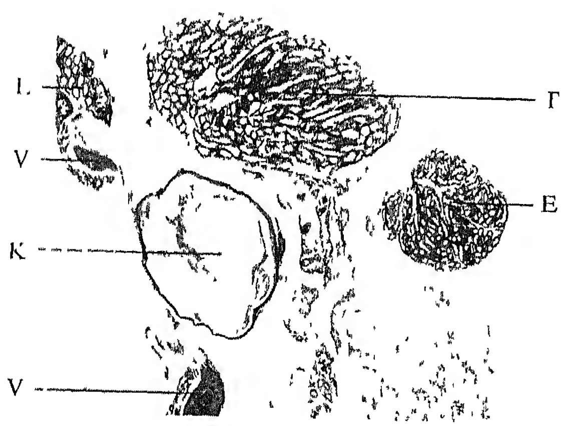


FIG 7

(*Facing p* 186)

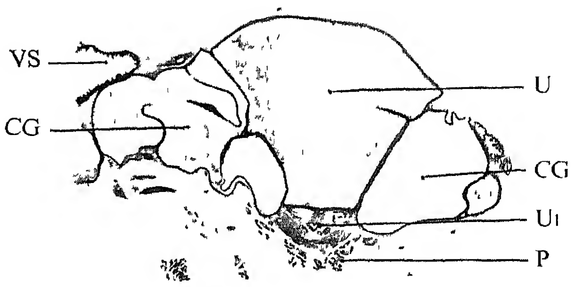


Fig 5

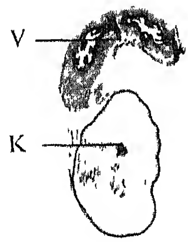


Fig 6

Fig 4

Fig 8

administration of oestrogenic compounds These structures have been present in two situations, namely (1) just dorsal to the prostatic urethra, projecting towards the abdominal cavity between the lower ends of the vasa deferentia, and (2) in the neighbourhood of the epididymis Seventeen instances of these abnormal structures have been observed among 342 mice treated with oestrogens, similar structures have not been seen in mice not so treated. Epithelial metaplasia with keratinization in the generative organs is a usual consequence of the prolonged administration of oestrogens to male mice The abnormal keratinized structures described may perhaps be remnants of the Müllerian duct which have been brought to view owing to metaplastic changes induced by the oestrogenic compounds.

References

Burrows, H., and Kennaway, N M (1934) 'Amer J Cancer,' vol **20**, p 48
Burrows, H (1935) 'Amer. J Cancer,' vol **23**, p. 490

Description of Plates 28 and 29

These represent the organs of mice treated with the oestrogens named below for the periods stated in days The significance of the lettering is as follows.

B, Bladder, *CG*, Coagulating gland; *E*, Epididymis, *ED*, Ejaculatory duct, *G O*, Genital omentum, *K*, Vestigial tubules or cyst adjacent to the vas deferens or epididymis, *P*, Prostate, *T*, Testis; *U*, Vestigial cyst adjacent to the urethra, *Ur*, Urethra, *V*, Vas deferens, *VS*, Vesicula seminalis

Fig. 1 Oestrone, 157 days Mouse 886

Fig. 2 Oestriol, 173 days *U*, Vestigial cyst measuring 13 × 13 × 10 mm, *V*, Vas deferens entering cyst-wall in which it descends to form the ejaculatory duct. Mouse 976.

Fig. 3 Oestrone Methyl Ether, 264 days Just above the urethra are the two ejaculatory ducts. Mouse 104. × 4

Fig. 4 Oestrone, 97 days. Mouse 856 × 12.

Fig. 5 Oestrone, 206 days. The ejaculatory ducts lie between the urethra and the vestigial cyst. Mouse 164. × 4.

Fig. 6 Oestrone, 61 days. Keratinized vestigial cyst attached to the vas deferens Serial sections show the cyst, *K*, to be completely closed Mouse 378. × 12

Fig. 7 Equilin, 90 days. *K*, Keratinized cyst attached to the vas deferens Mouse 361. × 12

Fig. 8 -Oestrone, 203 days. *E*, Head of epididymis with which is incorporated a body *K* consisting of tubules lined with squamous keratinizing epithelium Attached to the tail of the epididymis at its junction with the vas deferens is a cyst, *K*, 3 mm in diameter, lined with squamous keratinized epithelium and distended with keratinous debris. Mouse 305 × 12.

612 015 33

The "Continuing" Metabolism of Nitrogen in Animals

By H Borsook and G. L Keighley

(From the William G Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California, U.S.A)

(*Communicated by T H. Morgan, Foreign Member Royal Society—Received June* 21, 1935)

1—Introduction · The "Continuing" Metabolism

It has been known since the time of Voit that in passing from a high to a low level of nitrogen intake, or vice versa, there is a lag, which in rats, for example, may last as long as a month before equilibrium at the new low level is established Martin and Robison (1922) pointed out that the logarithm of the difference between the daily nitrogen excretion at the low level, designated as x, and the daily excretion in the interval between the two levels, a, *i e*, log $(a-x)$, plotted against time gives a straight line. The same relationship is demonstrable in the data of the experiment of Deuel, Sandiford, Sandiford, and Boothby (1928) This logarithmic relation indicates that the decomposition of the nitrogen represented by $(a-x)$, which we shall term the continuing metabolism, resembles a first-order reaction

The following question arises out of this phenomenon, which, so far as the writers are aware, has been given little attention. "Is the continuing metabolism suspended during the period when nitrogen equilibrium is being maintained by the ingestion of the larger quantity of protein, or does it persist at a rate indicated by the extrapolated value of log $(a-x)$ to zero time?" If the second alternative be true, it follows that in the state of nitrogen equilibrium there is storage of a portion of the exogenous nitrogen corresponding to the extent of the continuing metabolism.

A direct experimental attack on this problem appears to be excluded. It seemed that an approximate answer may be obtained indirectly by an experiment of which the argument is as follows. If the total protein metabolized on any one day is for the most part, if not entirely, of exogenous origin, it follows that when nitrogen equilibrium is maintained with a non-sulphur containing nitrogenous substance that the sulphur excretion will promptly fall to the endogenous level , and on the resumption of the normal diet it will again correspond to the sulphur intake On

the other hand, if there is a large continuing metabolism, the sulphur excretion will be above the endogenous level on a sulphur-free diet according to the extent of the continuing metabolism On the resumption of the normal diet the sulphur excretion on the succeeding days will be less than that excreted on the days prior to the interruption, in spite of the ingestion of the full quota of sulphur in the diet, because of the depletion of the store on which the continuing metabolism subsists

The experiment was carried out as follows. Men were maintained on a constant diet until the urinary nitrogen and sulphur excretion were constant The regime was then interrupted by a twenty-four-hour period when the original diet was changed for one in which the nitrogen was supplied with little or no sulphur After equilibrium was re-established with the original diet, there was a second twenty-four-hour interruption by a period of nitrogen starvation but adequate caloric intake The sulphur excretion on the first experimental day is a measure of the extent of the continuing metabolism on that day, and, compared with the day of nitrogen starvation, indicates the extent to which the continuing metabolism is normally in operation

The data on the nitrogen excretion on the days succeeding the nitrogen starvation and experimental days also contribute to a decision on this question. The lag in attaining a nitrogen balance in passing from one level of nitrogen intake to another, and the conformance of the data to a first-order reaction indicate that the rate of the continuing metabolism is proportional to the extent of the available store of metabolizable nitrogen. This store is greater at a higher than at a lower level of protein intake. If the nitrogen katabolized is derived mainly from the protein ingested on that day, the effect of a day of protein starvation should be noticeable only on that day The resumption of the diet on the following day should be accompanied by nearly the same nitrogen excretion as on the day preceding the day of starvation. On the other hand, if there is a large continuing metabolism the effects of such an interruption will appear not only on the day of the experiment but also, on the resumption of the original diet, in the quota of the continuing metabolism of the succeeding days

These experiments were undertaken originally for the purpose of obtaining an estimate of the fraction of the specific dynamic action of protein to be attributed to the stimulation of endogenous metabolism One of the hypotheses offered in explanation of the specific dynamic action of amino acids and protein is that amino acids in increased concentration in the tissues stimulate the general endogenous metabolism The experimental evidence presented in support of this hypothesis is the increase in urinary uric acid and sulphate following the administration of amino acids (Lewis,

Dunn, and Doisy, 1918) (Kiech and Luck, 1931–32) We have repeated these experiments and have confirmed in general the observations of Mendel and Stehle (1915) that the ingestion of protein is followed by an increase in urinary uric acid, and the observations of Lewis, Dunn, and Doisy (1918) on the similar effect of amino acids In the crucial experiment on the effect of ingested ammonium salts our results were the reverse of that obtained by Lewis, Dunn, and Doisy, and lead to the conclusion that the observed effects of protein and amino acids are not to be ascribed to a stimulation of the endogenous or continuing nitrogen metabolism. These experiments, undertaken for the purpose of measuring the stimulation of endogenous metabolism, led to the conclusion that no such stimulation occurs. This conclusion was confirmed by numerous observations on the sulphur excretion after the ingestion of amino acids and protein.

There is an additional point of interest in the ammonia experiments The quantities ingested—7·5 to 14 gm of nitrogen—are, so far as we are aware, the largest which have been ingested by men in so short a time. five to seven hours The results demonstrated the enormous capacity of the liver for converting ammonia to urea On the days when these relatively enormous quantities of ammonia were ingested the ammonia in the urine was only slightly increased above the normal limits. This, combined with the rapid increase in urea excretion, and the absence after two hours of any symptoms of distress, show that the liver allowed very little ammonia to pass into the systemic circulation.

2—Experimental Procedure

The subjects in all the experiments reported here were young men. Their daily diet was identical throughout the whole course of the experiment, except on the experimental days. Before beginning an experiment the subject was brought into nitrogen and sulphur balance, judged by the constancy of the urinary excretion at the desired level. When the balance had been maintained for a number of days, one experimental day was interposed ; the standard diet was then resumed, and was not interrupted by another experimental day until balance had been regained and maintained for several days.

The diet, consisting of only morning and evening meals, was as follows: breakfast: sugar, 24 gm; butter, 20 gm, ground beefsteak, fried, 200 gm; bread, 45 gm; cream (20%), 30 gm; fruits (oranges, grapefruit, or peaches), 210 gm, cereal, 140 gm; orange juice, 100 gm The evening meal: vegetables (cucumbers, asparagus, lettuce, celery, tomatoes, spinach, or

cauliflower), 125 gm; potatoes, 150 gm, sugar, 32 gm; butter, 28 gm, bread, 25 gm, fruits (oranges, grapefruit, peaches, or apples), 250 gm, orange juice, 200 gm; mayonnaise, 15 gm. Water, from 8 in the morning until 8 in the evening, 200 cc every hour

The urine was analysed for total nitrogen by the method of Kjeldahl, ammonia nitrogen by aeration, uric acid by a modification of the method of Morris and Macleod (Keighley and Borsook, 1934), and total sulphur by Robison's (1922) modification of Benedict's reagent (1909) with the preliminary precipitation of phosphate by the method of Fiske (1921).

The stools were not collected in any of these experiments, as the records in the literature show that changes in the diet are followed by only small changes in the amount of faecal nitrogen In these experiments, where the dietary regime was interrupted for only one day, it was extremely unlikely that any significant changes could have been detected in the stools

3—Experimental Evidence of the Continuing Metabolism

Table I is an abstract of some typical results on four subjects Of the two alternatives proposed above the data are definitely in favour of the persistence of the continuing metabolism. The clearest evidence is obtained from the figures on the sulphur excretion This was practically the same on the days of nitrogen starvation as when the nitrogen losses were covered by ammonia or glycine Table I also shows that ammonia, glycine, and an incomplete protein like haemoglobin can for one day supply most of the nitrogenous requirement.

In the other test of the existence of the continuing metabolism—the persistence for one or more days afterwards of the effect of one day's interruption in an otherwise constant dietary regime—the results obtained, though uniformly positive also, were more variable. The effects expected could hardly be large at best, and their trend was to some extent obscured by uncontrollable variations in the daily urinary nitrogen and sulphur excretion. These variations became especially marked in subjects 1 and 2 after they had been on the experimental regime for about six weeks. From then on fasting days or worse (sodium pyruvate, sodium bicarbonate, and succinic acid were taken on different days) were interposed five times in twenty-one days The nitrogen and sulphur excretion during the twenty-four-hour period immediately following such a day were larger than on the two subsequent days, though they were all three distinctly lower than on the days preceding the fast. It is improbable that a steady nutritional state was attained at this time in the intervals between the

TABLE I—THE EFFECT OF ONE DAY'S INTERRUPTION OF A CONSTANT DIETARY REGIME ON THE URINARY TOTAL NITROGEN AND TOTAL SULPHUR (24-HOUR PERIODS)*

Day No.	Subject 1 Diet	Subject 1 Total N	Subject 1 Total S	Subject 2 Diet	Subject 2 Total N	Subject 2 Total S
		gm	mg		gm	mg
1	Mixed diet ...	11·0	695	Mixed diet ...	12 5	759
2	,, ..	10·9	623	,,	12 8	784
3	,, ...	10 7	667	,, .	12 8	727
4	$(NH_4)_2CO_3$ (11 gm N) + 430 gm sucrose	12 9	373	$(NH_4)_2CO_3$ (7 5 gm N) + 400 gm sucrose ..	11 2	344
5	Mixed diet .	9·8	596	Mixed diet ..	9 8	669
6	,, .	9 5	537	,,	10 7	634
7	,,	10 6	703	,,	9 8	728
8	,,	11 6	685	,,	10 9	700
9	,, .	10 5	653	,,	10 8	738
10	430 gm sucrose	6 8	356	400 gm sucrose	6 4	303
11	Mixed diet .	7 5	519	Mixed diet .	8 4	602
12	,, .	8 9	612	,, .	11 0	647
13	,, .	9 3	565	,, ..	9 2	596
14	,, .	9 5	646	,, .	9 7	675
15	,, ...	8·8	602	,,	10 1	671
16	,, . ..	10·4	678	,, . .	10 2	666
17	Glycine (10 gm N) + 400 gm sucrose	11 8	315	Glycine (10 gm N) + 627 gm sucrose	11 1	271
18	Mixed diet .	11 3	528	Mixed diet	11 7	577
19	,, .	10 2	634	,,	11 1	660
20	,, . .	10 1	670	,, ...	10 4	685
21	,, ...	9 5	608	,, ...	10 0	631
22	,,	9 7	603	,, ...	11·2	674

* In our first experiments with haemoglobin there appeared to be more sulphur in the 24-hour urine than the sum of the quantity given with the haemoglobin plus the sulphur excretion on the nitrogen-free day From this we drew the conclusion that there may be some small stimulation of endogenous metabolism (Borsook and Keighley, 1935) Since then re-determination of the sulphur content of the haemoglobin showed that the original estimates were too low The method used first, digestion with perchloric and nitric acids, does not oxidize all the sulphur, and agreement between duplicates is not good Higher values with the duplicates agreeing within ± 1 per cent were obtained only after combustion in a bomb with an oxygen pressure of thirty atmospheres Employing these high values, the sulphur excreted on the haemoglobin days was not greater than the sum of that ingested plus the excretion on the nitrogen-free day. There is no evidence in these data, therefore, of a stimulation of endogenous metabolism We are indebted to Dr. H M Huffman for assistance with the combustions.

TABLE I—(continued)

Day No.	Subject 1 Diet	Total N gm	Total S mg	Subject 2 Diet	Total N gm	Total S mg
23	Haemoglobin (11 gm N) 387 mg S 400 gm sucrose	12 3	734	Glycine (10 gm N) 162 gm sucrose	10 7	313
24	Mixed diet .	11 3	645	,, .	13 5	690
25	,, .	10 7	612	,, .	11 7	717
26	,,	10 6	645			
27	,, .	10 5	611			
28	,, .	10 5	649			
	Diet discontinued for two weeks					
1	Mixed . .	11 1	780			
2	,, ...	11 7	690			
3	,, ..	11 4	725			
4	,, . .	11 6	717			
5	Neutralized pyruvic acid, 16 gm of acid	8·9	495			
6	Mixed	10 3	671			
7	,, . .	9 8	633			
8	,, .	9 5	676			
9	$NaHCO_3$, 31 gm .	7 9	488			
10	Mixed	10 9				
11	,, ...	9 1				
12	,, ...	8 8				

Day No.	Subject 3 Diet	Total N gm	Total S mg	Subject 4 Diet	Total N gm	Total S mg
1	Mixed .	11 1	723	Mixed . .	15·5	1149
2	,, . .	10 4	669	,, ..	14 9	1040
3	,, ...	11 3	751	,, .	16 1	997
4	,, ...	11·2	654	,, ...	16·8	1044
5	$(NH_4)_2CO_3$; 11 3 gm N.... . .	13·0	369	,, .	16 8	1070
6	Mixed	11 5	621	,, .	15 6	950
7	,, .. .	11 0	735	Beef, N = 10 8 gm, S = 681 mg—Breakfast .	15 2	916
8	,, ...	11 9	747	Haemoglobin, N = 10 7 gm, S = 310 mg—Breakfast	16 6	751
9	,, ...	10 4	659	Mixed . .	14 0	922
10	,, . . .	10 7	701	No breakfast . .	9 2	502
11	,, ...	10 7	646	Mixed	12 7	858
12	Fasting	6 5	342	,, . .	13·9	913
13	Mixed	9·6	604	,,	14 5	899
14	,, ...	10 4	735	,, .	15 1	995

Changes were made only in the breakfast, the evening meal being identical

TABLE I—(continued)

Day No	Diet	Subject 5 Total N gm	Total S mg
1	Breakfast Beef, N = 6 0 gm, S = 400 mg .	11·2	772
2	Mixed	12 5	748
3	,, . .	11 4	732
4	Breakfast haemoglobin, N = 6 0 gm, S = 120 mg	11 4	569
5	. .	11 1	753
6	.	10 8	676
7	Breakfast glycine, N = 6 0 gm	11 3	541
8	.	11 1	656
9	No breakfast .	9 6	501

Changes were made only in the breakfast, the evening meal being identical every day

experimental days. We are inclined, therefore, to place more weight on the results obtained during the first month when there was only one day in which no nitrogen was taken and none of complete fasting

In the experiment with haemoglobin the sulphur excretion was the sum of the sulphur content of the haemoglobin and the excretion on the day of nitrogen starvation. The sulphur in the haemoglobin (horse) used here was probably in a form not available for use by the organism. It appears to have been excreted too quickly to have undergone any metabolism. Analysis of the hourly excretion indicated that practically all of the haemoglobin sulphur was excreted in the six hours after ingestion. Against the possibility that this represented a stimulation of endogenous metabolism are the data in Table I showing that whereas the haemoglobin sulphur is completely excreted in 24 hours approximately twice this amount of sulphur taken in the form of vegetables and meat yields less than half its sulphur in the same period under similar conditions

Results similar to those in Table I were obtained on four other subjects with ammonia, alanine, glycine, and haemoglobin.

The alternative interpretation stated above—that when physiologically incomplete nitrogen is ingested the nitrogen metabolized is the sum of the endogenous (Folin) quota plus the nitrogen administered—may appear

to have some support in the negative nitrogen balances obtained with ammonia, glycine, and haemoglobin But the negative balances observed are too small to afford any strong support for this *a priori* improbable interpretation, especially as it appears to be definitely excluded by the sulphur figures If the ingestion of ammonia or glycine had completely suppressed the continuing metabolism the sulphur excretion on these days should have been much lower than on the day of nitrogen starvation; because the nitrogen and sulphur metabolism on the first day of nitrogen starvation are far above the endogenous levels

Our interpretation of this evidence therefore is that the continuing metabolism does not stop when food is taken In a man in nitrogen equilibrium at a urinary level of 10–11 gm daily, 50 to 60% of this nitrogen is derived from the continuing metabolism The remainder is the exogenous quota, *i e.*, derived from the nitrogen ingested on that day When physiologically incomplete nitrogen is ingested it may be expected that this quota will be a little larger than with a complete protein, because the extent of resynthesis (*see* below) into labile nitrogen will be somewhat less. As a result, there will be a small negative nitrogen balance This negative balance should be less than the figure for the daily endogenous metabolism When large quantities of single amino acids are taken the negative nitrogen balance may be augmented by the escape of a considerable quantity of the amino acid into the urine unmetabolized. For example, in the glycine experiments, Table I, the free amino nitrogen in the urine in subject 1 was 830 mg, and in subject 2 973 and 749 mg, whereas the normal figures were of the order of magnitude of 200 mg daily.

This conclusion regarding the extent of the continuing metabolism rests on the assumption that the daily quantitative changes in sulphur metabolism are not widely divergent from that of the nitrogen in the mixture of amino acids undergoing metabolism. The warrant for this assumption is that there is a lag in the excretion of sulphur as well as of nitrogen in passing from one level of protein intake to another. The question whether the sulphur is bound in an especially labile moiety, Wilson (1925), is beyond the scope of the present discussion.

In view of the lag in attaining nitrogen equilibrium in passing from one level of protein intake to another it would have been surprising if different results from those in Table I had been obtained They are practically different examples of the same lag In the formulation of theories of protein metabolism this well-known phenomenon, which is quantitatively more important than the endogenous metabolism, for example, has not been given the attention it deserves.

4—Urinary Uric Acid after the Ingestion of Ammonium Salts

Tables II to VI are summaries of experiments designed originally to measure the extent of the stimulation of endogenous metabolism by amino acids and proteins by the increases in urinary uric acid The procedure in these experiments was to administer the substances under investigation at least 24 hours after the last ingestion of purine containing food. A number of days later the identical procedure of the experimental day was repeated, except for the omission of the substance under investigation as the control The diet was that described above. We found that in spite of the ingestion of purines in the meat the urinary uric acid values 24 hours after the last meat meal were as low as those given in the literature for individuals maintained for long periods on a purine-free diet

In the experiments with ammonia, Tables II and III, 7 5 to 14 gm of nitrogen were ingested in the form of ammonium carbonate This salt was chosen because with it there can be very little disturbance of the acid-base balance after the ammonia is converted to urea. After a good deal of experimentation we finally settled on the following method of ingesting the ammonia. A saturated solution of ammonium carbonate was prepared, heated, and while hot was mixed with an equal volume of 3% agar which was allowed to gel Portions of the jelly containing 1 to 1·5 gm of nitrogen were swallowed each hour, washed down with a drink of cane sugar solution or water. There was, as a rule, some nausea at the end of the first hour, which lasted for another hour after the ammonia ingestion was begun There was no headache. All the subjects found that their powers of concentration were reduced for the first four hours while the ammonia was being taken. There were no symptoms of distress afterwards.

In most of the experiments sucrose was also taken in order to provide the caloric requirements. Unfortunately the sucrose solutions proved to be as distasteful or even more so than the ammonia gel. As a result, the quantity taken was not always sufficient for the caloric requirement. Nevertheless, it is improbable that this invalidated or beclouded the interpretation of the results obtained, as the control period was strictly comparable except for the omission of the ammonia, or other substance under investigation, and the subjects rested on a couch or in a chair throughout the day. Further, Gigon (1911) found that the feeding of

sugar alone left the nitrogen the same as on a fasting day. In one experiment the ammonia was taken without sucrose On the control day for this experiment only agar and water were taken

Similar results to those in Tables II and III were obtained with three other subjects.

The urinary uric acid was different on the two types of control days When sucrose was taken there was a morning rise attaining a maximum at about 1 p m , after which it declined. This was observed earlier by Mendel and Stehle (1915). On a fasting day the morning values were fairly constant There was a decline in the afternoon, and became quite low between 7 p m. and 11 p m At no time throughout the day did the values rise above those in the 8 a m. to 10 a m. period These differences in the control days must be borne in mind in making comparisons with the experimental days.

TABLE II—INCREASES IN URINARY URIC ACID FOLLOWING THE INGESTION OF AMMONIUM CARBONATE

SUBJECT 1

Time	Ammonia + sucrose: Urine volume	Ammonia + sucrose: Total urinary nitrogen	Ammonia + sucrose: Uric acid	Sucrose: Urine volume	Sucrose: Total urinary nitrogen	Sucrose: Uric acid
	cc	mg	mg	cc	mg	mg
6- 9 a m	570	1280	53	630	1320	54
9–11	330	945	50	710	931	42
11 a.m.–1 p.m.	650	1270	54	400	858	56
1 - 3	350	1290	61	745	755	50
3– 5	690	1750	61	695	639	43
5– 7	995	1650	52	268	535	24
7–11	880	2650	93	526	1155	44
11 p.m –8 a.m	440	2870	68	392	1479	71
Total .	4905	13705	492	4366	7672	384
Following 24 hours	1490	9760	371	1450	7500	355

Ammonia + sucrose: The ingestion of ammonia began at 9 and finished at 4 230 gm sucrose in 1600 cc water between 9 and 4. 200 gm sucrose in 1000 cc water between 7 p m. and 11 p m

Sucrose: 230 gm sucrose in 1600 cc water and the same amount of agar as on the ammonia day (150 cc, 1·5%) between 9 a.m. and 4 p m. 200 gm sucrose in 1000 cc water between 7 p.m. and 11 p.m.

TABLE II—(continued)

Time	Ammonia without sucrose: Urine volume	Ammonia without sucrose: Total urinary nitrogen	Ammonia without sucrose: Uric acid	Fasting: Urine volume	Fasting: Total urinary N	Fasting: Uric acid	Fasting: Urine volume	Fasting: Total urinary N	Fasting: Uric acid
	cc	mg	mg	cc	mg	mg	cc	mg	mg
7– 9 a m	300	716	34	570	822	35	485	693	31
9–11	168	683	35	370	695	39	455	656	35
11 a m – 1 p m	290	735	31	350	703	39	205	628	26
1– 3	240	970	40	265	677	35	225	620	28
3– 5	258	1280	43	208	609	24	218	606	26
5– 7	318	1870	37	196	619	25	132	613	23
7–11	1018	4130	70	395	1240	54	375	1234	44
11 p m – 8 a.m	760	6140	103	452	2330	83	790	2703	92
Total	3352	16524	393	2806	7695	334	2885	7753	305

Ammonia without sucrose: Water as on fasting days Ammonia ingestion began at 1 p.m, finished at 7 p m, with 200 cc water each hour 11 0 gm N.

Fasting: 150 cc water each hour from 7 a m to 1 p m., 200 cc hourly from 1 p m to 7 p m, 500 cc between 7 p m and 11 p m.

Time	Ammonium acetate: Urine volume	Ammonium acetate: Total nitrogen	Ammonium acetate: Uric acid	Acetic acid: Urine volume	Acetic acid: Total nitrogen	Acetic acid: Uric acid
	cc	mg	mg	cc	mg	mg
6– 9 a m.	375	1580	49	610	1450	45
9–11	770	1200	37	830	929	29
11 a m –1 p m	522	1740	61	730	931	48
1– 3	470	1830	59	530	932	44
3– 5	534	1910	53	615	765	34
5– 7	385	1560	33	620	761	34
7–11	520	2300	44	560	1480	39
11 p m –8 a m	375	3090	96	530	2030	91
Total	3951	15210	432	5025	9278	364

Ammonium acetate: 55 1 gm crystalline ammonium acetate + 140 gm cane sugar dissolved in 100 cc 2% agar, ingested between 9 30 and 5 30 Between 9 a m. and 10 p m. an additional 500 gm of cane sugar. Total water = 2000 cc. N = 10 gm.

Acetic acid: 42 9 gm acetic acid + 200 gm sucrose dissolved in litre of water ingested in portions at hourly intervals between 9 a.m. and 6 p m. During the day another 300 gm of sucrose were ingested. Total water = 2000 cc.

The data show clearly that in every case an increased excretion of uric acid followed the ingestion of the ammonia. The interpretation of this result is postponed until after the presentation of the results with amino acids. The only point that will be discussed here is whether or not the increased uric acid excretion can be ascribed to the diuresis, *i e*, that it is

Table III—Increases in Urinary Uric Acid Following the Ingestion of Ammonium Carbonate

Subject 2

Time	Ammonia + sucrose			Sucrose		
	Urine volume	Total nitrogen	Uric acid	Urine volume	Total nitrogen	Uric acid
	cc	mg	mg	cc	mg	mg
6– 9 a m.	410	1750	56	190	1510	46
9–11	320	1024	37	540	1210	34
11 a.m.–1 p m	124	818	40	485	1050	41
1– 3	520	1400	57	473	785	32
3– 5	430	1320	45	192	545	20
5– 7	440	1510	42	188	532	21
7–11	680	2630	63	234	766	37
11 p m.–8 a m.	290	1950	54	253	1110	52
Total	3214	12402	394	2555	7508	283
Following 24 hours	1130	9840	437	1200	8360	367
	Ingestion of ammonium carbonate in 110 cc 1 5% agar begun at 9 a m and finished at 1 p m 300 gm sucrose, 2000 cc water between 9 a m and 7 p m. 100 gm sucrose in 1000 cc water between 7 p.m. and 11 p m N = 7 5 gm.			110 cc 1 5% agar, 300 gm sucrose, 2000 cc water between 9 a m and 7 p m. 100 gm sucrose in 1000 cc water between 7 p m and 11 p m		

a "washing out" effect. This interpretation seems to be definitely excluded by the almost identical excretion between 11 p.m. and 8 a m in every case on the experimental day and control day If there had been any "washing out" it might be expected that there would have been a compensatory decline on the ammonia days below the control figures when the diuresis was over. Further, the total volume of urine was not much less on the control than on the experimental days, and in the experiment with ammonium acetate and acetic acid was actually 1070 cc greater

on the control day. Inspection of the tables shows that there is no correlation between urine volume and uric acid excretion The uric acid excretions on the days succeeding the experimental and control days give no indication of greater washing out on the days when ammonia was taken. Similarly Chaikoff and Larson (1935) found no causal relation between diuresis and allantoin and uric acid excretion.

5—Urinary Uric Acid after the Ingestion of Amino Acids, Protein, Sodium Pyruvate, and Sodium Bicarbonate

Tables IV, V, and VI are extracts of typical results in a few of the many experiments designed to obtain from the urinary nitrogen, uric acid, and sulphur some quantitative estimate of the stimulation of endogenous metabolism by proteins and amino acids These experiments were a repetition, with some variations, of those reported by Mendel and Stehle (1915), Lewis, Dunn, and Doisy (1918), and Gibson and Doisy (1923).

One of the difficulties in experiments such as these is that the early-morning-fasting uric acid values at times are distinctly above the figures usually obtained. On these days the uric acid excretion may remain high, even when fasting, throughout the day. This, as Lewis, Dunn, and Doisy observed, occurs even when the subjects are maintained for a long period on a non-purine diet. In the experiments reported here only those experimental and control days were selected for comparison where the early-morning fasting values were nearly identical on the control and experimental days

Table IV gives the results of two experiments with proteins. For the gelatine experiment there is no comparable fasting day ; but the rise following the ingestion of this protein is so striking as to exclude all doubt regarding its effect. The same result was obtained in every one of six other experiments with gelatine. The increase in uric acid with haemoglobin in four experiments was always less than with gelatine. This protein, which is exceedingly difficult and unpleasant to ingest in any quantity, was chosen on account of its low sulphur content The results in these experiments are in accord with those obtained by Mendel and Stehle

Our results with amino acids, with some exceptions, were the same as those obtained by Lewis, Dunn, and Doisy. An example of the occasional exception which occurred is shown in the first group of data in Table V. In the first experiment the result was clearly negative In the second, in which the glycine was given morning and evening, the uric acid excretion in the morning was no greater than on the fasting day ; in the evening there was a marked increase. The possibility of there having been only

TABLE IV—EFFECT OF INGESTION OF PROTEINS ON URINARY URIC ACID EXCRETION

SUBJECT 2

Time	Gelatine		
	Urine volume	Total nitrogen	Uric acid
	cc	mg	mg
6– 8 a m	59	738	40
8–10	425	1526	87
10–12	750	2128	84
12 noon– 2 p m	269	1751	56
2– 4	202	1530	45
4– 6	246	1409	41
Total	1951	9082	353

69 gm gelatine in 215 cc water between 8 a m and 9 a m 100 cc water hourly from 10 a m to 1 p m, and 120 cc hourly from 2 p m to 5 p m.

SUBJECT 1

Time	Haemoglobin + sucrose			Sucrose		
	Urine volume	Total nitrogen	Uric acid	Urine volume	Total nitrogen	Uric acid
	cc	mg	mg	cc	mg	mg
6– 9 a m	660	1170	56	630	1320	54
9–12	835	1730	77	910	1360	70
12 noon– 3 p.m.	420	2370	79	945	1184	77
3– 6	455	2150	59	829	917	55
6–11	875	2980	62	660	1428	34
11 p m–8 a m	640	3040	77	392	1479	71
Total ..	3885	13440	410	4366	7878	361
Following 24 hours	2380	11520	414	1450	7500	355
	75 gm haemoglobin (11 gm N) between 9 and 11 a m Sucrose and water were the same as on the day when sucrose was taken alone			Between 9 a m and 4 p m 230 gm sucrose in 1000 cc water. Between 7 and 11 p m 200 gm sucrose and 1000 cc water		

TABLE V—EFFECT OF INGESTION OF AMINO ACIDS ON THE URIC ACID EXCRETION

SUBJECT 2

Time	Glycine + sucrose			Glycine with and without sucrose			Sucrose		
	Urine volume	Total nitrogen	Uric acid	Urine volume	Total nitrogen	Uric acid	Urine volume	Total nitrogen	Uric acid
	cc	mg	mg	cc	mg	mg	cc	mg	mg
6– 9 a m	327	1587	51	390	1572	48	181	1400	43
9–12 noon	520	1540	40	350	1630	59	782	1735	54
12 noon– 3 p m	225	1910	47	430	1880	51	715	1310	52
3– 6	270	2240	46	130	990	23	336	811	31
6–11	520	2860	44	600	2840	71	378	1032	47
11 p.m. 8 a m	235	1990	37	270	2930	65	253	1110	52
Total	2097	12127	265	2170	11842	317	2645	7398	279
Following 24 hours	1760	11680	292	1820	13540	390	1200	836	367

Glycine + sucrose: 55 gm glycine were taken in 5 portions between 9 a m and 1 p.m 162 gm sucrose were ingested by 1 p.m Between 9 a.m. and 6 p.m 1500 cc water ingested

Glycine with and without sucrose: 27 5 gm glycine were taken at 9 a.m and another 27·5 gm at 7 p.m 16 gm of sucrose taken hourly between 9 a m. and 6 p.m with 100 cc water No sugar taken after 6 p m. Another 300 cc water between 7 p m. and 11 p m.

Sucrose: 162 gm sucrose in 1600 cc water ingested between 9 a.m and 4 p m. Sucrose, however, all ingested by 1 p m

SUBJECT 3

Time	Glycine			Fasting		
	Volume urine	Total nitrogen	Uric acid	Urine volume	Total nitrogen	Uric acid
	cc	mg	mg	cc	mg	mg
6– 8 a m	233	648	23	575	1099	40
8–10	76	679	72	395	845	47
10–12 noon	681	1340	75	370	737	33
12 noon– 2 p.m	483	1420	44	291	730	32
2– 4	68	840	36	260	738	35
4– 6	117	984	27	158	630	33
Total. .	1658	5911	277	2049	4779	220

Glycine: 32 3 gm of glycine (5·75 gm N) ingested between 8 and 9 a m. on experimental day. Water otherwise same as on fasting day

Fasting: 250 cc water at 6 a m., 300 cc at 8 a m , 200 cc hourly at 9, 10, 11, 12, and 1, and 100 cc at 2, 3, 4, and 5.

TABLE V—(continued)

SUBJECT 1

Time	Mixture of amino acids			Fasting			Fasting		
	Urine volume	Total N	Uric acid	Urine volume	Total N	Uric acid	Urine volume	Total N	Uric acid
	cc	mg	mg	cc	mg	mg	cc	mg	mg
7– 9 a.m	655	808	34	485	693	31	570	822	35
9–11	275	696	35	455	656	35	370	695	39
11 a m – 1 p m	280	676	31	205	628	26	350	703	39
1– 3	170	703	38	225	620	28	265	677	35
3– 5	160	1360	56	218	606	26	208	609	24
5– 7	153	1320	29	132	613	23	196	619	25
7–11	1020	3070	60	375	1234	44	395	1240	54
11 p.m – 8 a.m	860	4785	98	790	2703	92	452	2330	83
Total .	3573	13418	381	2885	7753	305	2806	7695	334

SUBJECT 2

Time	Mixture of amino acids			Fasting		
	Urine volume	Total N	Uric acid	Urine volume	Total N	Uric acid
	cc	mg	mg	cc	mg	mg
7– 9 a.m.	92	776	33	330	1090	37
9–11	280	930	33	115	547	27
11 a m.– 1 p m	380	882	32	365	1020	36
1– 3	140	520	23	190	781	27
3– 5	147	1010	39	61	357	16
5– 7	185	1450	31	92	648	17
7–11	940	3300	56	275	1194	36
11 a m – 8 a.m.	440	2930	65	385	1855	39
Total .	2604	11798	312	1813	7492	235
Following 24 hours	2020	12630	396	1630	11400	416

TABLE V—(continued)

The following mixture of amino acids was ingested between 1 and 3 by each of the two subjects above :—

	gm		gm
glycine	12 00	l-tyrosine .	6 5
d-l-alanine	14 35	d-arginine . .	3 75
d-glutamic HCl	11 7	l-leucine .	8·4
l-aspartic HCl	10 58	haemoglobin . . .	6 8

The mixture contained 10 gm of nitrogen It was partly dissolved and suspended in water and neutralized with NaOH 150 cc water were taken each hour from 7 to 1 inclusive At 4 p m 200 cc, 5 p m 200 cc, 6 p m 200 cc, and between 6 and 11 500 cc water On fasting days the same amount of water was ingested without the amino acids

Very thirsty between 4 p m and 6 p m. Mouth parched in spite of the ingestion of large quantities of water No other symptoms of distress.

an apparent reduction in urinary uric acid, because of interference by the amino acids in the urine with the colorimetric determination of uric acid, is excluded because the uric acid was first precipitated with zinc, and then assayed—essentially the method used by Christman and Mosier (1929), by which these authors accounted for the negative results obtained by Zwarenstein (1928). There were increases in urinary uric acid in the two experiments where the mixture of amino acids was ingested. This was a neutralized mixture containing aspartic and glutamic acids. It is possible that a fraction of the increase in uric acid followed on the excretion of the base after the amino acids were metabolized (*see* the results given below with sodium pyruvate and sodium bicarbonate) But it is unlikely that this was responsible for more than a small fraction, as the same increase in uric acid was obtained in subject 2, where there was no increased excretion of sulphur in the urine, the usual consequence in our experiments of an increased ingestion of base (Tables VIII and IX)

The experiments in Table VI were carried out to examine this possibility—that the increase in urinary uric acid after the ingestion of sodium pyruvate observed by Gibson and Doisy (1923) was an effect of the base rather than of the acid radical. In Gibson and Doisy's experiments the controls were fasting days We compared the uric acid excretion on the day on which pyruvic acid was ingested with two types of control days, one a fasting day the other in which the same amount of base was ingested in the form of sodium bicarbonate as was taken with the pyruvate. The results obtained show that, as Gibson and Doisy found, the excretion of uric acid is greater with sodium pyruvate than on a fasting day , but it is not significantly different from the bicarbonate control day There

is possibly some slight stimulation by the pyruvate, but the main effect appears to be one of the base

The interpretation we have placed on the results of the experiments summarized in Tables II to VI is that the increases in urinary uric acid observed represent increased formation of uric acid from ammonia, ingested as such, or arising from the deamination of the amino acids. This conclusion is, of course, not yet completely proven. It appears to be the simplest hypothesis, and it is in accord with all the data known to us There is no *a priori* reason for excluding from man a residuum of the mechanisms whereby the bulk of the nitrogen is handled in birds In the Dalmatian coach dog a considerably larger fraction of the nitrogen is handled in this form, so that changes in the excretion of urea and uric acid run parallel (Larson and Chaikoff, 1935) Even if only a special amino acid such as histidine can serve as a non-purine precursor of uric acid, an additional atom of nitrogen is necessary for the formation of the purine ring. In view of the rapid fluctuations in the urine it seems probable that some of this uric acid is formed in the kidney It has been demonstrated that uric acid is formed in the kidney in the rat (Borsook and Jeffreys, 1935) Krebs (1933) showed that glycine, alanine, aspartic acid, and glutamic acid are rapidly deaminized in the kidney In the liver ammonia is very rapidly converted to urea Ammonia arising from the deamination of amino acids in the kidney is more available, therefore, for uric acid formation wherever it may occur in the body than ammonia in the portal blood Hence the need for large quantities of ammonia This hypothesis accounts for the close parallel between the rise in urinary uric acid and the increase in caloric metabolism after the ingestion of a non-purine containing protein such as gelatine (Borsook and Keighley, 1934) in the absence of any stimulation of endogenous metabolism; for the increases in uric acid obtained with such different amino acids as glycine, alanine, glutamic and aspartic acids ; for the higher uric acid excretion on a higher than on a lower non-purine protein diet , and for the observation of Lewis and Doisy · "That there is no evidence that in man a diet high in protein and high in its content of arginine and histidine increases the uric acid excretion over that eliminated on a high protein diet low in these amino acids" (Lewis and Doisy, 1918)

The negative result of Lewis, Dunn, and Doisy (1918) with ammonium chloride may be ascribed to the small amount of ammonia given—1 97 gm —too small to permit the escape from the liver of a sufficient quantity for uric acid synthesis elsewhere, and possibly also to the acidosis induced by the ammonium chloride

Table VII shows the great capacity of the liver for removing ammonia

TABLE VI—THE EFFECT OF PYRUVIC, SUCCINIC ACID, AND SODIUM BICARBONATE ON THE URINARY URIC ACID

SUBJECT 1

Time	Fasting*			Fasting + $NaHCO_3$†		
	Urine volume	Total N	Uric acid	Urine volume	Total N	Uric acid
	cc	mg	mg	cc	mg	mg
7– 9 a m	485	693	31	410	843	39
9–11	455	656	35	160	681	32
11 a.m – 1 p m	205	628	26	342	720	32
1– 3	225	620	28	133	635	34
3– 5	218	606	26	98	533	28
5– 7	132	613	23	185	723	27
7–11	375	1234	44	235	1205	59
11 p m.– 8 a m.	790	2703	92	595	2940	132
Total	2885	7753	305	2158	8280	383

Time	Sodium pyruvate‡			Succinic acid§		
	Urine volume	Total N	Uric acid	Urine volume	Total N	Uric acid
	cc	mg	mg	cc	mg	mg
7– 9 a.m.	260	680	32	610	727	38
9–11	470	820	36	515	613	40
11 a m.– 1 p m.	490	860	40	350	614	33
1– 3	165	610	39	329	655	36
3– 5	150	690	41	212	627	36
5– 7	147	660	40	274	629	30
7–11	190	1180	168	730	1420	62
11 p m – 8 a m	850	3580	138	680	2110	84
Total	2722	9080	434	3700	7395	359

* Fasting. From 6 a m to 1 p m 100 cc water each hour, from 1 p m to 7 p.m. 200 cc water each hour

† Fasting + $NaHCO_3$. Water same as in *. At 1 p m, 7 gm $NaHCO_3$, 2 p.m., 6 gm, 4 p m., 9 gm, and 5 p m, 9 gm ingested.

‡ Sodium pyruvate 6 a.m to 1 p m, 100 cc water each hour; 1 p.m, 16 gm neutralized freshly distilled pyruvic acid in 165 cc water, at 3.40 same. 100 cc water each hour from 6 a m to 1 p.m 200 cc from 1 p m to 7 p m., 500 cc between 7 p m. and 11 p m

§ 6 a m to 1 p.m, 100 cc water each hour. From 1 p.m and to 4 30 p m., in four portions, 40 gm neutralized succinic acid in 1150 cc water Before 11.30 and after 5 30, 100 cc of water each hour from 6 a.m to 11 p.m

TABLE VI—(continued)

SUBJECT 4

Time	Fasting + $NaHCO_3$‖ Urine volume	Total N	Uric acid	Sodium pyruvate¶ Urine volume	Total N	Uric acid
	cc	mg	mg	cc	mg	mg
7– 9 a m	290	928	48	390	1080	37
9–11	88	689	39	390	858	40
11 a m.– 1 p m	115	820	46	490	1040	36
1– 3	65	539	35	180	896	45
3– 5	0	0	0	137	786	37
5– 7	155	1242	54	107	740	31
7–11	245	1537	74	170	1590	91
11 p.m.– 8 a m.	300	3300	122	265	2370	76
Total	1258	9055	418	2129	9360	393

‖ 6 a m to 1 p.m , 100 cc water each hour At 1 p.m , 7 gm $NaHCO_3$, 200 cc water , 2 p m., 6 gm+125 cc water ; 5 p m , 9 gm+200 cc water , 6 p.m , 9 gm+200 cc water.

¶ 6 a.m. to 1 p m , 100 cc water each hour 16 gm neutralized freshly distilled pyruvic acid in 150 cc water at 1 p.m 16 gm + 150 cc water at 6 p.m. 100 cc water hourly from 6 a m to 11 p.m.

from the portal blood and converting it to urea. The ammonia excretion on days when ammonia was ingested was very little more than on the corresponding control days. When the same quantity of nitrogen was taken as glycine, over approximately the same length of time, the urinary ammonia nitrogen was, in Subject 1, 547 mg against 381 mg on the control day, and in Subject 2, 384 and 430 mg on the two different glycine days

TABLE VII—URINARY AMMONIA ON DAYS WHEN AMMONIA WAS INGESTED COMPARED WITH CORRESPONDING CONTROL DAYS

Subject	Ammonia nitrogen ingested	Ammonia nitrogen excreted on Ammonia day	Fasting day
	gm	gm	gm
1	11 0	424	381
1	10·0	488	459
2	7·5	381	213
3	11 0	397	390
4	14 0	439	357
5	18 0	516	474

against 213 mg on the control day As a result of the more extensive formation of ammonia in other tissues than the liver, presumably by deamination of part of the glycine in the kidney, a larger amount escaped in the urine than when all the ammonia passed first to the liver The increased quantities of amino nitrogen in the urine, 830 mg in Subject 1, 973 and 749 mg in Subject 2, attest to the escape of these amino acids from the liver and their passage through the kidney

6—Urinary Total Sulphur After the Ingestion of Amino Acids, Protein, Sodium Pyruvate, and Sodium Bicarbonate

Tables VIII, IX, and X contain data on urinary sulphur following the ingestion of ammonia, amino acids, and haemoglobin The hourly changes give no clear evidence of an increased sulphur excretion (and hence of stimulation of endogenous or continuing protein metabolism) comparable with the rise in metabolism or urinary uric acid. The totals for the whole experimental period are similarly negative In the mixture of amino acids used in two experiments there was a small quantity of haemoglobin containing 40 mg of sulphur. This sulphur appears to have been excreted very quickly. In order to compare the sulphur excretion with the control days 40 mg must be subtracted from the figure for the interval from 1 p.m to 11 p m. With this figure subtracted the difference between the amino acid and control days is small, and could easily have been the result of the large amount of base ingested. In Subject 2 there was no increase over the whole period.

The ingestion of sodium bicarbonate on a fasting day led to an increased excretion of sulphur The base, therefore, taken with the sodium pyruvate, appears to be responsible for the increased excretion of sulphur as well as of uric acid observed with this substance The increased excretion of sulphur when sodium aspartate was administered to rats, Kiech and Luck (1931–32), may similarly have been an effect of the base rather than of the amino acid radical.

There is no evidence, therefore, either in the data on sulphur or uric acid excretion, that the ingestion of amino acids is followed by a stimulation of tissue protein metabolism.

7—The Continuing Metabolism and the Extent of the Synthetic Processes Normally in Operation

The data in Table II indicate that the continuing metabolism is always in operation and constitutes a large fraction of the total nitrogen meta-

TABLE VIII—INFLUENCE OF AMINO ACIDS, AMMONIA, SODIUM PYRUVATE, AND SODIUM BICARBONATE ON THE TOTAL URINARY SULPHUR

SUBJECT 1

Time	Total Urinary Sulphur on day of: Sucrose only mg	Sucrose + glycine mg
6– 9 a m		55
9–12 noon		54
12– 3 p.m		50
3– 6		38
6–11		53
11 p m – 8 a m		103
Total	397	353

On both days 230 gm sucrose between 9 a m and 4 p m in 1600 cc water 200 gm sucrose in 1000 cc water between 7 p m and 11 p m On glycine day, in addition to above, 55 gm glycine in five portions between 9 a m. and 1 p m

Time	Total Urinary Sulphur on day of: Fast mg		Fast mg		Ammonia mg		Amino acid mixture containing 40 mg of S. mg	
6– 9 a m	58		60		51		54	
9–11	36		35		38		37	
11 a m.– 1 p m	30		35		35		36	
1– 3	29	363	34		39		40	
3– 5	40		31	275		241	60	385
5– 7	36		32				53	
7–11	71		73				94	
11 p.m – 8 a.m.	187		139				178	
Total	487		439		404		552	

On all days 150 cc water each hour from 7 a m to 1 p m. inclusive, 200 cc each hour from 1 p m to 7 p m 500 cc water between 7 p m and 11 p m Ammonia from 1 p m to 7 p.m Neutralized amino acid mixture from 1 p m to 4 p m.

Time	Total Urinary Sulphur on day of: Fast + $NaHCO_3$ mg*		Sodium pyruvate mg†	
6– 9 a m	46		53	
9–11	36		38	
11 a.m – 1 p m	36		42	
1– 3	42	393	38	389
3– 5	40		42	
5– 7	49		38	
7–11	91		70	
11 p m – 8 a m	171		201	
Total.. .	511		522	

On both days 100 cc water from 6 a m. to 1 p m, 200 cc water each hour from 1 p m to 7 p m 500 cc water between 7 p m and 11 p.m

* $NaHCO_3$ as follows 1 p m., 7 gm, 2 p m, 6 gm, 4 p m, 9 gm, 5 p m, 9 gm

† Sodium pyruvate as follows: 1 p m, 16 gm freshly distilled pyruvic, neutralized, in 165 cc water, 3.40 p m, same

TABLE IX—INFLUENCE OF AMINO ACIDS, AMMONIA, SODIUM PYRUVATE, AND SODIUM BICARBONATE ON THE TOTAL URINARY SULPHUR

SUBJECT 2

Total Urinary Sulphur on day of

Time	Sucrose	Sucrose + ammonia	Sucrose + glycine		Sucrose + glycine	
	mg*	mg†	mg‡		mg§	
6– 9 a m	77	91	97		87	
9–12 noon			44*		58*	
12– 3 p m.			39		43	
3– 6	278	314	32	235	27	289
6–11			60		65*	
11 p m – 8 a.m.			60		96	
Total	355	405	332		376	

*† 300 gm sucrose in 2000 cc water between 9 a m. and 7 p.m ; 100 gm sucrose in 1000 cc water between 7 p.m. and 11 p m

‡ 55 gm glycine in five portions and 162 gm sucrose between 9 a m. and 1 p m. 1500 cc water between 9 a m. and 6 p.m.

§ 27 5 gm glycine at 9 a.m and at 7 p m 16 gm sucrose + 100 cc water hourly between 9 a m and 6 p m. inclusive. 300 cc water between 7 p.m. and 11 p.m.

Total Urinary Sulphur on day of

Time	Fast	Alanine		Mixture of amino acids—40 mg S
	mg*	mg†		mg‡
7– 9 a m.	67	91		58
9–11	33	45		38
11 a m –1 p m.	40	39*		32
1– 3	31	32		25*
3– 5	21	23		43
5– 7	31	27		50
7–11	66		168	82
11 p.m –8 a m.	138			99
Total .	427	425		427

* 150 cc water from 7 a m to 1 p.m inclusive, 1000 cc between 1 p.m. and 6 p m ; 500 cc between 6 p m and 11 p m.

† Water same as *, between 11 a m and 12 noon, 25 gm alanine (dl) in three portions = 3 93 gm N

‡ Water as in *, mixture of amino acids between 1 p.m. and 3 p.m

bolized, even when nitrogen equilibrium is maintained by the ingestion of protein. It is obvious that the continuing metabolism does not have the " wear and tear " significance of Folin's endogenous metabolism. Its extent is directly proportional to the level at which the nitrogen balance has been set by the previous dietary history, and is to some extent, though not entirely, independent of the nitrogen intake on that day.

TABLE X—INFLUENCE OF PYRUVIC ACID ON THE TOTAL URINARY SULPHUR

SUBJECT 9

	Total Urinary Sulphur on day of	
Time	Fast + $NaHCO_3$ mg*	Sodium pyruvate mg†
7– 9 a.m	49	53
9–11	42	40
11 a.m – 1 p m	47	46
1– 3	40	51
3– 5	92 (3–7)	43
5– 7		40
7–11	102	118
11 p m – 8 a m.	232	178
Total.	604	569

* 6 a m to 1 p m , 100 cc water each hour. 1 p m —7 gm $NaHCO_3$ + 200 cc water, 2 p m —6 gm $NaHCO_3$ + 125 cc water, 5 p m —9 gm $NaHCO_3$ + 200 cc water; 6 p m —9 gm $NaHCO_3$ + 200 cc water. 100 cc water from 6 p m to 11 p m.

† 6 a m to 11 p.m , 100 cc water each hour. 16 gm freshly distilled pyruvic acid, neutralized, in 150 cc water at 1 p.m. Same at 6 p m

No information can be gained from the data in Table II regarding the form of the nitrogen upon which the continuing metabolism subsists Fortunately, there are abundant data in the literature on this point. All observers are agreed that the free amino nitrogen content of the tissues remains constant through extreme variations in diet and nutritional state (Greene, 1919) (Mitchell, Nevens, and Kendall, 1922) The data of van Slyke and Meyer (1913–14) and Wishart (1915) particularly indicate that six hours after a protein meal the free amino nitrogen of the tissues is at the fasting level. But the urinary nitrogen does not return to the fasting level for nearly 24 hours after a large meat meal (Williams, Riche, and Lusk, 1912) The greater amount of basal nitrogen excreted on a high protein diet than on a low, and the lag in attaining equilibrium in passing from one level of nitrogen intake to another are examples of the same phenomenon It follows from these findings and the logarithmic relation-

ship between the continuing metabolism and time that the nitrogen which supports the continuing metabolism must exist in the tissues in some form other, and presumably more complex, than amino acids. Otherwise the free amino nitrogen on a low non-protein diet should be less than on a high protein diet It follows also that on any one day a quantity of exogenous nitrogen corresponding to the extent of the continuing metabolism is converted into this more complex form. Van Slyke and Meyer (1913–14) were unable to find any increase in polypeptide nitrogen in the tissues of a dog after a large intravenous injection of amino acids. From this they concluded that retained amino acids are not stored as such but are converted into protein The chief difficulty in the way of accepting this hypothesis is that feeding a protein deficient in sulphur does not change the composition of the proteins of the liver (Lee and Lewis, 1934), the organ in which protein storage most readily occurs (Seitz, 1906, Gautier and Thiers, 1928; Gautier, 1929, 1931, 1933, Addis, 1935), nor in the muscles At the same time the large changes in the urinary nitrogen-sulphur ratio with changes in the diet show that the composition of the labile nitrogen, which is the store of nitrogen supporting the continuing metabolism, does vary in its nitrogen-sulphur ratio (Wilson, 1925; Cathcart and Burnett, 1925–26; Deuel, Sandiford, Sandiford, and Boothby, 1928) Nevertheless, the continuing metabolism is proof that storage of nitrogen does occur, and the findings are conclusive that this nitrogen is not stored in the form of amino acids. In spite of the above difficulty, therefore, it seems a warrantable conclusion that the nitrogen is stored as polypeptides or protein or both.

We are therefore led to a conception of normal protein metabolism which differs in an important respect from the generally accepted theory first enunciated by Folin According to Folin's theory, anabolic processes, such as the synthesis of protein, are restricted in the adult organism in equilibrium to not more than the replacement of the " wear and tear " or endogenous quota This quota corresponds to not more than the minimum excretion of nitrogen on a diet containing little or no nitrogen, but otherwise adequate. When more than this minimal quantity of protein nitrogen is ingested the excess over the endogenous requirement is considered to be stored in the tissues as amino acids. These are quickly metabolized, and the nitrogen appears in the urine chiefly as urea. According to our view, the abundant data in the literature on nitrogen storage, the lag in attaining equilibrium in passing from one level of nitrogen intake to another, observations such as those in Table II, and the data on the constancy of the free amino nitrogen of the tissues, indicate that the anabolic processes continually in operation must be more

extensive than postulated in the current theory of protein metabolism, and in men may normally amount to 50% or more of the nitrogen intake.

It follows from this that the extent of the continuing metabolism in different animals, under comparable conditions of nitrogen intake, carbohydrate metabolism, and work will be a function of the extent of the synthetic processes normally in operation This need not be the same in all animals, and may be a characteristic of the species The amount of nitrogen which can be stored therefore, and *pari passu* the extent of the continuing metabolism, need not be the same in all animals.

The continuing metabolism in the normal dietary state in man is quantitatively more important than the endogenous metabolism postulated by Folin If the metabolism which leads to the urinary creatinine be excluded it is an open question whether the remainder of the endogenous metabolism yielding urea, ammonia, and part or possibly all the uric acid has any physiological reality We approach, therefore, the point of view of Mitchell, Nevens, and Kendall (1922) that under normal dietary conditions there may be no breakdown of tissue But the hypothesis proposed here differs from that of Mitchell, Nevens, and Kendall in the same respect as it does from Folin's original theory in postulating much more extensive synthetic processes continually in operation.

8—Thermodynamical Considerations of the Constancy of the Amino Acid Concentration in the Tissue

Thermodynamical considerations of the dynamic steady state between protein and amino acids in the tissues lead to the same conclusion that this balance can only be maintained by extensive synthetic processes. The work of van Slyke and Meyer (1913–14), Wishart (1915), Greene (1919), Mitchell, Nevens, and Kendall (1922) have established beyond doubt the actuality of this balance This balance is not, as in a true thermodynamic equilibrium, the result of an equalization of the reactions proceeding to the "right" and "left," *e g*,

$$A + B \rightleftharpoons C + D,$$

because this true equilibrium is much farther over on the side of breakdown than the steady state *in vivo*.

The following examples will illustrate this point In the hydrolysis and synthesis of protein and polypeptides the equilibrium position varies according to the mode of hydrolysis With pepsin the degree of hydrolysis at equilibrium is less than with trypsin, or with a poly- or dipeptidase. In all cases the physiological state is much farther over on the protein side of

the reaction than the thermodynamic equilibrium state. When the co-ordination of metabolism is destroyed, *e g.*, by maceration, autolysis promptly sets in. To obtain a numerical expression of this result we may consider the hydrolysis and synthesis of a dipeptide. Dr. Huffman has determined in this laboratory the free energy of formation of leucylglycine The value is only approximate at present and refers to the solid state, but it will serve for purposes of the present discussion. From this value the free energy change $-\Delta F$, computed for the reaction

$$\text{leucylglycine} + H_2O \rightarrow \text{leucine} + \text{glycine},$$

is 3970 calories, giving an equilibrium constant

$$\frac{(\text{leucine})\ (\text{glycine})}{(\text{leucylglycine})} = 800 \text{ (approximate)}$$

The degree of hydrolysis α will be a function of the concentration C It may be computed from the equation $C\alpha^2/(1-\alpha)$. Inserting the value for K of 800 at initial concentrations of dipeptide 10, 1, and 0·01 molal, the values of α are 0 987, 0 997, and 1 000 respectively. For any conceivable concentration *in vivo* the equilibrium position is far over in the direction of nearly complete hydrolysis. The situation is not materially different for higher peptides.

Another example is the synthesis of alanine from ammonia and pyruvic acid. The free energy change $-\Delta F$ for the oxidative deamination of alanine

$$CH_3\ CH\,(NH_3^+)\,COO^- + 1/2\,O_2 \rightarrow CH_3\,.\,CO\,.\,COO^- + NH_4^+,$$

when all the reactants are taken at 1 molal activity, and at 25° C, is approximately 39,000 calories From this value the equilibrium relationship is

$$\frac{(\text{Pyruvate}^-)\ (NH_4^+)}{(\text{Alanine}^\pm)\ (O_2)^{1/2}} = K = 10^{29\ 3}$$

(Borsook and Huffman, 1933, Wurmser and Mayer-Reich, 1933 *a*, *b*). The physiological steady state is again enormously removed from the state of thermodynamic equilibrium, although mechanisms both for the synthesis and oxidative deamination of alanine exist *in vivo*.

With amino acids the equilibrium position is not always so far over on the side of deamination. In the formation of aspartic acid from fumaric acid and ammonia, 1 mg % of ammonia and 116 mg % of fumarate would be in equilibrium with 8 mg % of aspartic acid (Borsook and Huffman, 1933).

It is probable, however, that where the reaction is between keto acids and ammonia the equilibrium position is always far over on the side of deamination.

Since the physiological steady states and the thermodynamic equilibrium positions are usually, if not always, so far apart it is misleading to write the *in vivo* reactions as they are sometimes done,

$$\text{Tissue protein} \rightleftharpoons \text{amino acids} \rightleftharpoons \text{deaminized residues} + NH_4.$$

The small increases in concentration which are physiologically possible cannot *per se* reverse the reaction in the direction of the synthesis. The reactions from right to left, the syntheses, absorb relatively large amounts of energy, of the order of 5000 to 10,000 calories per mol of reactants Special mechanisms for the transfer of this energy must intervene in order to carry them through. The nature of these mechanisms and some of the laws governing them have been discussed in detail elsewhere (Borsook, 1935) It is sufficient to point out here that hydrolysis and synthesis of protein, deamination and synthesis of most amino acids, under physiological conditions, cannot normally be simple reversions of the same reactions The fact that a steady state does exist *in vivo*, that this state is in the direction of synthesis, far from the thermodynamic equilibrium, in spite of the abundance of hydrolytic and oxidative enzymes demonstrably in operation, *e.g.*, the continuing metabolism, makes it extremely probable that the synthetic and breakdown reactions are different, and that the balance between them is maintained through the intervention of other reactions. The more variable factor in this balanced system will be the rate of synthesis, because it cannot proceed spontaneously but is dependent on other metabolic processes in the cell

9—The Stimulation of Endogenous or Continuing Metabolism by Amino Acids

In considering a stimulation of tissue metabolism as a possible explanation for all or part of the specific action of protein, no distinction has hitherto been made between endogenous metabolism (in Folin's sense) or continuing metabolism Employing the urinary creatinine—Folin's criterion—as a measure of endogenous metabolism, most of the evidence indicates that there is no stimulation of this fraction of the nitrogen metabolism after the ingestion of protein or amino acids, *i e.*, there is no immediate increase in urinary creatinine Our own observations with alanine are in accord with those of Lewis, Dunn, and Doisy (1918). The

only observers who have found an increase in urinary creatinine on the same day in which an increased quantity of protein was ingested are Beard and Barnes (1931–32). Their observations are at variance with the original observations of Folin on the independence of the urinary creatinine of the amount of protein in the diet, which have been repeatedly confirmed Beard and Barnes did not ascribe the increases in urinary creatinine which they observed to stimulation of the endogenous metabolism.

Under the conditions in which experiments on the specific dynamic action are commonly carried out, the initial or basal nitrogen metabolism is predominantly continuing metabolism The data in Tables II to X indicate that there is little if any significant stimulation of this metabolism after the ingestion of amino acids or protein

This conclusion is supported by other evidence. If in an animal in nitrogen equilibrium the ingestion of protein or amino acids is followed by a stimulation of the endogenous or continuing metabolism, a depression below the level at the beginning of the experiment should occur later when the drive of the absorbed amino acids has spent itself. There is no evidence in any of the experiments reported in the literature or in our own of such a depression in the urinary nitrogen, uric acid, or sulphur data. Gigon (1911) referred to experiments in the older literature and some of his own in which a depression of the caloric metabolism was observed some hours after the ingestion of a small quantity of protein. This point does not appear to have been supported by adequate controls and there are no references in it to the later literature.

Another fundamental datum similarly argues against any general stimulation of metabolism as a factor responsible for the specific dynamic action of protein. If there is any considerable stimulation of tissue metabolism the heat evolved in excess of the basal should always be greater per gram of extra nitrogen metabolized than the physiological heat of combustion of the protein burned. Nearly all the values in the literature on the specific dynamic action of protein and amino acids, expressed as a ratio of calories in excess of the basal to nitrogen excreted in excess of the basal, are only one-third to one-half the physiological heat value of the nitrogen ingested. It is obvious that all of the carbon of the ingested protein is not burned, or there is a sparing of tissue carbon. The general tissue stimulation hypothesis therefore loses all its force as an explanation for the increase in caloric metabolism.

It becomes exceedingly improbable in the absence of any stimulation of the general metabolism of the tissues that an increase in concentration of amino acids in the tissues stimulates an increased metabolism of similar

material already there; especially as these exogenous amino acids are themselves undergoing metabolism Experiments on tissue slices provided some direct evidence on this point. When individual amino acids or a mixture were added to the Ringer's solution in which slices of liver, kidney, or spleen, or sections of nearly intact diaphragm, or small intestine of the rat were maintained no increased breakdown was observed of tissue protein, peptides, or amino acids. If anything, there were indications of reverse processes (Borsook and Jeffreys, 1935). So far as the specific dynamic action of protein is concerned, this observation gains in significance from the demonstration that the specific dynamic action of protein is independent of the action of the ductless glands, and is of the same order of magnitude in their absence as in the normal animal (Dubois, 1916; Nord and Deuel, 1928; Gaebler, 1929; Dann, Chambers, and Lusk, 1931–32; Fulton and Cushing, 1932; Johnston, 1932; Daggs and Eaton, 1933).

It is characteristic of the present uncorrelated state of the various aspects of the field of protein metabolism that a conflict between the fundamental postulates in two different parts should have passed unchallenged. In the calculation of the results in experiments on the specific dynamic action of protein the assumption is made tacitly that the basal nitrogen metabolism at the beginning of the experiment is continued throughout. This is opposed to the conception that in an animal in nitrogen equilibrium most of the nitrogen is derived from the protein ingested on that day, and only a small remainder is the endogenous metabolism of the tissues. If the latter is the condition which actually occurs, then when sufficient nitrogen is ingested for the maintenance of nitrogen equilibrium, the basal nitrogen metabolism must be suppressed, because it is mainly continuing and not endogenous metabolism, yet the basal nitrogen metabolism is assumed in the calculation of the results to have persisted unchanged throughout the experiment. The fact that the most concordant quantitative results have been obtained on the assumption of a constant basal (continuing) metabolism indicates that this assumption approximates more nearly of the two to the conditions which prevail (Borsook and Winegarden, 1931; Borsook and Keighley, 1934)

10—Ammonia as a Precursor of Urinary Uric Acid or Allantoin

The interpretation we have placed on the uric acid excretion following the ingestion of amino acids and protein is opposed to that of Folin, Berglund, and Derick (1924), and of Lennox (1925) According to these authors the increased uric acid excretion on a high protein diet (non-

purine) represents merely an increased excretion—not an increased formation of uric acid. Folin and his collaborators based their conclusion on their finding of a lower blood uric acid and a greater excretion of injected urate in men on a high protein diet than when the same subjects were on a low protein diet Their data were obtained from experiments on three individuals. In one on the high protein diet the plasma uric acid was 3 0 mg per 100 cc, and 87% of the injected urate was recovered in the urine On the low protein diet some months later the plasma uric acid was 4 2 mg %, and 73% of the injected urate was recovered in the urine. In another of these cases on the high protein diet the plasma uric acid was 3·4 mg % as against 5 to 5 4 mg % on the low protein diet, but only 74% of the injected urate was recovered in the urine on the high protein diet as against 91% on the low protein diet. In the third case there was little difference in the plasma uric acid or in the urinary excretion of injected urate on the high and low protein diets. The interpretation of their results on the excretion of injected urate is difficult because of the large variation in urinary uric acid on the control days (preceding the injection of uric acid) The conclusion of Folin, Berglund, and Derick (1924) is further weakened by their statement " that there is no definite relationship between the level of the uric acid and the magnitude of the uric acid excretion, even under practically normal conditions in most subjects, as we have failed to find any close relationship between the uric acid in the plasma and the expected elimination after the injection of uric acid."

Harding, Allin, and Van Wyck (1924–25) questioned the high blood uric acid figures obtained by Folin, Berglund, and Derick (1924) when their subjects were on a low protein, purine-free diet, 3·0, 5·4, 5·6, and 6·0 mg per 100 cc of plasma. In further disagreement are the observations of Larson and Chaikoff (1935, *a*, *b*), who found the blood uric acid in dogs raised when urinary uric acid was increased.

Lennox adhered to the explanation of Folin, Berglund, and Derick for the higher urinary acid on a high protein diet, viz., increased excretion without increased production. Yet his own observations point precisely to the explanation he sought to disprove In every one of his cases the ingestion of amino acids or protein was followed by a greatly increased excretion of uric acid, and in three of his cases by a distinct rise in blood uric acid In one case there is a striking example of the difference between increased production and elimination as compared with merely increased elimination. Administering 40 gm of glycine on the eighth day of a fast, there was a small rise in blood uric acid which persisted on the average for four days (from 9·2 to 9·5 mg %); at the same time the urinary uric acid rose from 188 mg to 663, 240, 453, and 349 mg on the four days

after the injection of the glycine At this stage atophan was administered, the blood uric acid fell from 10·3 to 4·57 and 5 37 mg % on the next two days; and after an excretion of 855 mg of uric acid at the end of the first day of the atophan the succeeding days were the lowest in the whole series of 14 days, 145 and 104 mg These and other similar results in Lennox's paper demonstrate, it seems, conclusively that proteins and amino acids lead to an increased production as well as elimination of uric acid in man

The following observations provide additional evidence that the increase in urinary uric acid cannot be considered as evidence of a stimulation of endogenous metabolism In men the administration of amino acids is usually followed by an increase in urinary uric acid In rabbits the reverse occurs, *i e*, the allantoin excretion is diminished (Christman and Lewis, 1923) Yet amino acids exert a specific dynamic action in both animals Again, from the experiments of Larson and Chaikoff (1935, *a*, *b*), who found that injections of insulin and epinephrine increase the urinary uric acid in dogs, it might be concluded that the increase in urinary uric acid in men following the ingestion of amino acids is the result of a stimulation of the adrenal medulla, which in turn is responsible for the general increase in metabolism This interpretation appears to obtain support from the observation of Reid (1934) that there is a slight lowering of the blood sugar (about 10 mg %) for about two hours after the ingestion of glycine, which condition may evoke an increased production of epinephrine; and from the direct observation of Nord and Deuel (1928) that glycine increases the epinephrine production in rabbits But, as pointed out above, in rabbits the administration of glycine leads to diminished excretion of allantoin And Nord and Deuel (1928) found that the specific dynamic action of glycine is the same in adrenalectomized as in normal dogs

The experiments with ammonia, Tables II and III, suggest a relation between carbohydrate metabolism and uric acid formation from ammonia. When sucrose was ingested there was a pronounced morning rise in uric acid which was absent on the fasting days Further, more urinary uric acid was excreted when sucrose was ingested with the ammonia than when the ammonia was taken alone These findings, so far as they go, parallel the recent findings on the synthesis of uric acid in birds (Schuler and Reindel, 1933) In these experiments it was found that two precursors are necessary, one nitrogenous, which may be ammonia, urea, or amino acids, and an unknown non-nitrogenous substance formed in the liver. In addition may be cited the well-known fact that carbohydrate feeding increases the urinary uric acid in men and allantoin in dogs (Cathcart, 1909; Umeda, 1915), and the observations of Larson and Chaikoff,

mentioned above, that insulin and epinephrine stimulate the production of uric acid and allantoin in Dalmatian and normal dogs.

Summary

A new term, the continuing nitrogen metabolism, is defined. It is the nitrogen metabolized on any one day which is already present in the tissues—distinguishing it from exogenous nitrogen It is not related to the endogenous, or "wear and tear" metabolism of Folin

It is shown that in man in nitrogen equilibrium at a level of 10–11 gm of urinary nitrogen daily, the continuing metabolism constitutes more than half the total urinary nitrogen. The extent of the continuing metabolism is a function of the previous dietary history, and among other factors is directly proportional to the level of the nitrogen intake

The data on the continuing metabolism, taken in conjunction with the constancy of the amino acids in the tissues, indicate that in an adult animal in nitrogen equilibrium extensive synthetic processes, involving amino acids, are continually in operation Thermodynamical data are presented supporting this conclusion.

Ammonia can serve as a precursor of urinary uric acid in man.

Analysis of the data on urinary uric acid and sulphur, and on the specific dynamic action of protein and amino acids, reveals that there is no stimulation of either the endogenous or the continuing nitrogen metabolism by amino acids.

References

Addis, T. (1935) Personal communication

Beard, H H, and Barnes, B. O (1931–32). 'J Biol Chem.,' vol. **94**, p. 49.

Benedict, S. R. (1909) 'J Biol Chem,' vol. **6**, p. 363.

Borsook, H (1935) 'Ergeb. Enzymforsch,' vol. **4**, p. 1

Borsook, H, and Huffman, H M (1933) 'J Biol. Chem,' vol **99**, p. 663.

Borsook, H., and Jeffreys, C E P (1935) 'J Biol. Chem.,' vol **110**, p 495.

Borsook, H, and Keighley (1934) 'Proc Nat Acad. Sci, Wash.,' vol. **20**, p. 179.

Borsook, H, and Winegarden, H M (1931). 'Proc. Nat. Acad Sci., Wash.,' vol **17**, p 75

Carthcart, E. P. (1909) 'J Physiol,' vol **39**, p. 311.

Cathcart, E P, and Burnett, W. A (1925–26). 'Proc. Roy Soc,' B, vol. **99**, p. 405.

Christman, A. A., and Lewis, H. B (1923) 'J. Biol. Chem,' vol. **57**, p 379

Christman, A. A, and Mosier, E. C (1929) 'J. Biol. Chem,' vol. **83**, p. 11

Daggs, R G, and Eaton, A. G. (1933) 'Amer J Physiol,' vol **106**, p 299

Deuel, H J, jr., Sandiford, I, Sandiford, K, and Boothby, W. M. (1928) 'J Biol Chem,' vol. **76**, pp 391 and 407

Dubois, E F (1916). 'Arch. Int. Med,' vol **17**, p 915

Dann, M., Chambeis, W H, and Lusk, G (1931–32) 'J Biol Chem,' vol **94**, p 511
Fiske, C H (1921) 'J Biol Chem.,' vol **47**, p 59
Folin, O, Beiglund, H, and Deiick, C (1924) 'J Biol Chem,' vol **60**, p 361
Fulton, M N, and Cushing, H (1932) 'Arch Int Med,' vol **50**, p. 649
Gaeblei, O H (1929). 'J Biol Chem,' vol **81**, p 41.
Gautier, Cl (1929) 'Bull Soc Chim biol,' vol **11**, p 168
—— (1931). *Ibid*, vol **13**, pp 143 and 147
—— (1933) *Ibid*, vol **15**, p 1563.
Gautiei, Cl, and Thiers, H P (1928) 'Bull Soc Chim biol,' vol **10**, p 537.
Gibson, H. V, and Doisy, E A (1923) 'J Biol Chem,' vol **55**, p 605
Gigon, A (1911) 'Aich ges Physiol,' vol **140**, p 509
Gieene, C W (1919) 'J Biol Chem,' vol **39**, p 435
Haiding, V J, Allin, K D, and Van Wyck, H B (1924–25) 'J Biol Chem,' vol **62**, p 61
Johnston, M W (1932) 'J. Clin Invest,' vol. **11**, p 437
Keighley, G, and Borsook, H. (1934). 'J Lab. and Clin Med,' vol **19**, p 650
Kiech, V. C., and Luck, J. M (1931–32). 'J Biol. Chem.,' vol **94**, p 433
Krebs, H A (1933) 'Z. physiol Chem,' vol **217**, p 191; vol **218**, p. 157
Laison, P S, and Chaikoff, I L (1935). 'J Biol. Chem,' vol **108**, p 457.
—— (1935). Vol. **109**, pp 85 and 395.
Lee, W. C, and Lewis, H B. (1934). 'J Biol Chem.,' vol **107**, p 649.
Lennox, W. G. (1925) 'J Biol Chem,' vol **66**, p. 521
Lewis, H B, and Doisy, E. A (1918). 'J Biol Chem,' vol **36**, p. 1.
Lewis, H. B, Dunn, M S., and Doisy, E A. (1918). 'J. Biol Chem.,' vol **36**, p. 9.
Maitin, C J, and Robison, R. (1922) 'Biochem. J.,' vol **16**, p 407.
Mendel, L. B, and Stehle, R. L (1915) 'J Biol Chem.,' vol **22**, p. 215
Mitchell, H H., Nevens, W. B, and Kendall, F R (1922). 'J. Biol. Chem.,' vol. **52**, p 417.
Noid, F., and Deuel, H J (1928) 'J. Biol Chem,' vol. **80**, p. 115
Reid, C J (1934). 'J Mental Sci,' vol **80**, p 379
Robison, R (1922) 'Biochem J,' vol **16**, p 134
Schuler, W, and Reindel, W (1933) 'Z physiol Chem,' vol. **221**, p 209
Seitz, W. (1906) 'Arch ges Physiol,' vol **111**, p 309
Umeda, N (1915) 'Biochem J,' vol **9**, p 421
van Slyke, D. D, and Meyei, G. M. (1913–14) 'J Biol Chem,' vol. **16**, p. 213
Williams, H B, Riche, J A, and Lusk, G. (1912) 'J Biol Chem,' vol **12**, p. 349
Wilson, H. E C. (1925, *a*) 'Biochem J,' vol **19**, p. 322
—— (1925, *b*) Vol **20**, p 76
Wishait, M B. (1915) 'J Biol Chem.,' vol **20**, p 535
Wuimsei, R., and Mayei-Reich, N (1933, *a*). 'C R Soc. Biol, Paris,' vol. **112**, p 1648
—— (1933, *b*) *Ibid*, vol **113**, p 244
Zwarenstein, H (1928) 'Biochem J.,' vol **22**, p 307

576, 312 3

Some New Evidence on the Physical Nature of Plant Nuclei from Intra-Specific Polyploids

By IRENE MANTON, B.A , Ph D., Lecturer in Botany in the University of Manchester

(*Communicated by W H Lang, F R S—Received June* 27, 1935)

[PLATES 30 TO 32]

INTRODUCTION

The object of this paper is to make clear certain facts regarding the resting nuclei and mitotic phenomena of plants These facts are of material importance to both experimental and morphological cytologists, but so far do not seem to have been clearly appreciated by either. Indeed, the extreme diversity of outlook between the two branches of what should be one science may easily be shown by a simple quotation from current text-books of each Thus Gray (1931), on p. 122 of his " Text-Book of Experimental Cytology," remarks: " Since all nuclei exhibit a visible granular or fibrillar structure after coagulative fixation, it is generally supposed that the structures seen as preserved preparations or in moribund nuclei are to be regarded as *purely artificial products of coagulation, which cannot be correlated with the fundamental structure of a living nucleus*. This view, developed many years ago by Hardy, is now accepted by the majority of animal cytologists " Sharp, on the other hand, in the new edition of the " Introduction to Cytology " (1934), on p 54, remarks: " These results [from plants], together with the analogous findings of Chambers on the prophases in animal spermatocytes, indicate that the reticulum appearing in a well-fixed nucleus *fairly represents a delicate thready structure actually present during life* and rendered more distinctly visible by fixation."

While admitting, as will be shown, the inadequacy of the " reticulum " conception as a complete description of any nucleus, it must be confessed that the entirely negative view of " the majority of animal cytologists " comes as nothing less than a shock to anyone familiar with the degree of precision and certainty of which plant cytology is becoming increasingly capable. This precision is generally recognized where it concerns the morphology and behaviour of chromosomes in the definitive state To deny all validity to the morphological approach to the resting

nucleus appears, in contrast, both unjustified and undesirable. It is undesirable, owing to the very obscurity of most nuclei in the living state; a state so difficult to observe and interpret that every possible source of information should be exploited. That such excess of caution is also unjustified may perhaps become more apparent from the new evidence to be presented. This admittedly deals with dead cells, but the type of reasoning is such that it does not depend for its validity on the detailed interpretation of fixation alone It may therefore help to bridge the gap between the morphological and experimental fields of observation.

Intra-specific polyploidy provides cytological material which is in many ways unique Cells of exactly comparable organs and tissues and of uniform genetical constitution, but of different chromosome number, may be compared, even in polyploid chimaeras, within the confines of a single individual. The method to be pursued is that of a comparative investigation of such material from several members of the Cruciferae and in one Liliaceous plant *Allium ursinum*. The immediate enquiry centres round problems concerning the nature of "prochromosomes" and the behaviour of nucleoli Both these subjects involve controversial morphological details, and for both data of unusual clarity have been obtained Exhaustive treatment of either subject is, however, purposely omitted, and discussion will be confined to those aspects which are most closely relevant to the new data By this means it is hoped to preserve the conciseness of the more general results which collectively refer to the nucleus as a whole

The Problem of Prochromosomes

The question of prochromosomes has been the subject of cytological papers from 1904 to the present day, but only the most significant contributions in an extensive bibliography need be quoted The earliest are those of Rosenberg who in 1904 and 1909 drew attention to the apparent coincidence between chromosome numbers and the number of chromatic bodies present on the surfaces of the nuclei of certain plants possessed of small chromosomes These bodies are the prochromosomes of this and subsequent workers * The tissues examined by Rosenberg included the suspensor and endosperm of *Capsella*, the tentacles of *Drosera*, the integuments of *Zostera*, *Calendula*, etc , and the facts were

* An alternative name, "euchromocentres," introduced by Gregoire, is also in current use This has the advantage that it brings the chromatic bodies of different kinds of nuclei into a uniform system of nomenclature The present work is, however, not concerned with this question of homology and therefore the older terminology has been preferred.

quoted in support of the then urgent topic of the persistence of chromosome individuality In modern times the most important paper is that of Doutreligne (1933) This writer, following the idea introduced as early as 1907 by Gregoire, studied (by various methods including that of Feulgen) and carefully figured the whole mitotic process in a number of prochromosomal plants She concluded that her prochromosomes did not merely represent the chromosomes in a general way but that they were precise portions of these, namely, the regions containing the spindle attachment constrictions.

The idea of prochromosomes as the organic representatives of the chromosomes in the resting state is thus based on two lines of evidence—determination of numerical correspondence and demonstration of persistence throughout the mitotic cycle Had only the works of Rosenberg and Doutreligne existed both might have been accepted as proved. The considerable technical difficulties involved has unfortunately obscured the issue by giving rise to a mass of conflicting literature This has been ably summarized in Doutreligne's historical introduction, and some of the causes for disagreement are obvious. The inherent uncertainty of interpretation of fixation effects in the crucial stages of prophase and telophase certainly accounts for some of the diversity of opinion on the subject of mitosis. On the numerical side, every writer, from Rosenberg onwards, has stressed the difficulty of counting accurately small bodies distributed on the surface of a sphere which at the same time contains an opaque nucleolus. In general only an approximate agreement is arrived at when a large number of cells is examined at random. This no doubt is the explanation of a very disconcerting situation Much of the work which has aimed at extension of Rosenberg's observations has merely shown that it is possible to demonstrate an apparent correlation between prochromosomes and chromosome counts which are either uncertain or even seriously wrong. Thus uncertainty inevitably attaches to a count based on only a single side view of a meiotic metaphase in an embryo sac (Malte, 1908), while errors so great as to give the number 24 instead of 30 for *Lunaria biennis* have actually been found in several examples from the classic " confirmatory " work of Laibach (1907) on the Cruciferae (Manton, 1932). Further enumeration of literature is unnecessary, for enough has been said to show the desirability of some new line of approach to the problem

The Resting Nucleus in some Polyploid Cruciferae

Conclusive evidence in favour of Rosenberg's hypothesis, for the Cruciferae, is afforded by polyploid material, and this also makes possible an

almost equally clear demonstration of the persistence of autonomy of the chromatic bodies through every stage of mitosis. A number of instances of polyploid races and polyploid chimaeras were reported in 1932 (Manton), and some of these have been intensively studied from other aspects since. For the present purpose almost any genus of the family would serve except for the few with large chromosomes such as *Hesperis* and *Matthiola*, but it will be sufficient to consider only two species, *Biscutella laevigata* L and *Iberis semperflorens* L

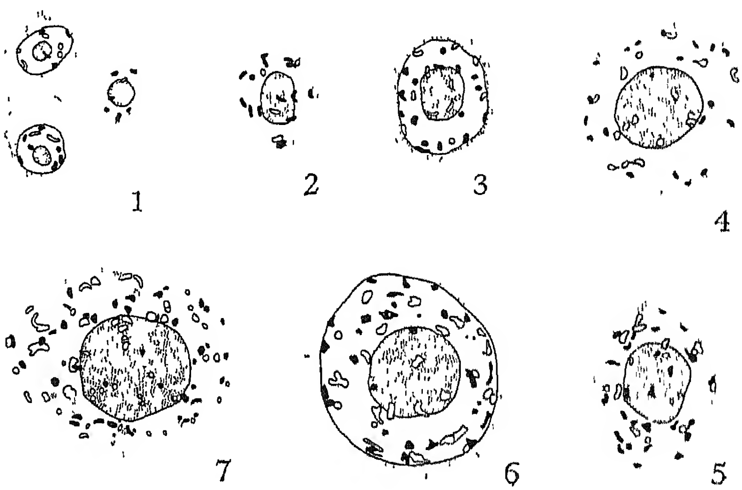
In *B laevigata* there are at present available individual plants with $2n$, $3n$, $4n$, $5n$, and $6n$ chromosomes, n being the original haploid number 9. The origin of the specimens is irrelevant to the present discussion and has been partly detailed elsewhere (Manton, 1934) It is sufficient to remark that the even polyploids (diploids, tetraploids, and hexaploids) were collected wild in various parts of Europe, while the odd polyploids (triploids and pentaploids) are the hybrids between them. Monoploid (" haploid ") tissue is only known from the products of meiosis in diploids

Photographs of dividing cells in all of these are shown at a magnification of × 2000 in figs. 17–22, Plate 30 The actual numbers represented are 9, 18, 27, 36, 45, and 54 A few chromosomes in the last are almost out of focus owing to the inevitable increase of photographic difficulty with high numbers. Every chromosome is, however, actually visible in each specimen, the accuracy of the mere counts is therefore indisputable.

A preliminary view of resting nuclei from each of the photographed preparations is given in figs 1–6 These are all drawn at the same magnification (× 2000) and from the point of view of prochromosomes; selection of the nuclei was at random, for the only criteria considered were that they should be uncut, should not be obviously in prophase or telophase, and should be as near as possible to the cell photographed. The general agreement between " prochromosomes " and the known chromosome numbers is striking. In the nuclei depicted for the first four members of the series (n, $2n$, $3n$, and $4n$ in figs. 1–4) the numerical correspondence happens to be exact In the other two the agreement is still close, but the observational difficulties previously referred to are exemplified Thus for the pentaploid, fig 5, only 42 bodies are drawn instead of the expected 45 In this case the discrepancy is possibly due to the nucleus being not quite intact, for insufficient allowance had been made for increasing cell-size and the section was somewhat too thin. For the hexaploid, fig 6, this defect was eliminated, but the selected nucleus proved difficult to interpret exactly owing to the presence of some apparently supernumerary bodies of very small size. The approximate count is, however, definitely in the right region (54).

The remaining drawing, fig 7, completes the preliminary survey by the addition of a $12n$ cell to the polyploid series. This is from a patch of chimaeral tissue in the root of a hexaploid. The original plant had actually $6n + 1$ chromosomes (*i e*, 55) and the chimaeral tissue contained one metaphase plate of 110 chromosomes In fig 7, 100 bodies are visible

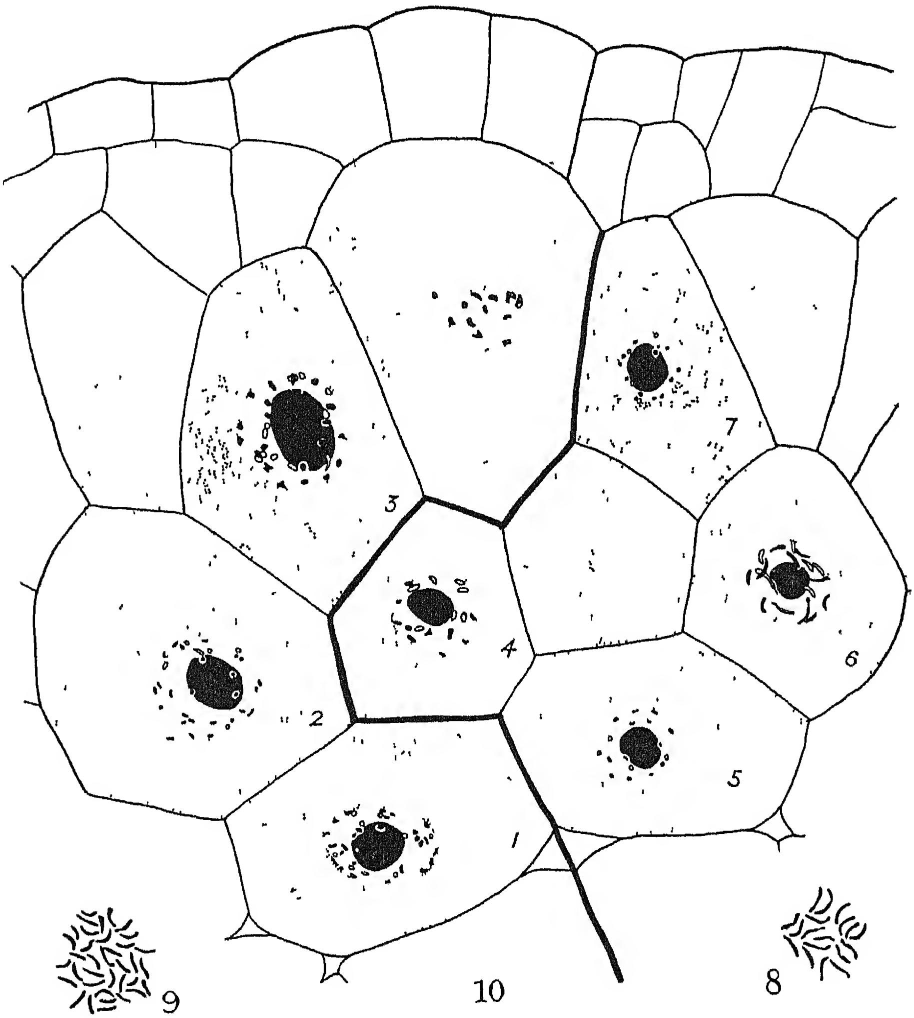
The evidence from the preliminary survey becomes more conclusive when it is supplemented by further observation of chimaeras. In addition to the $12n$ chimaera, three cases of tetraploid tissue in diploid plants of the same species, *B laevigata*, have been met with One of these



FIGS 1–7—Resting nuclei in polyploid *Biscutella laevigata L.* × 2000, from the preparations photographed in Plate 30, figs. 17–22 Fig 1, monoploid cells ("haploid") from a pollen tetrad ($n = 9$) Fig 2, diploid root. Fig. 3, triploid root Fig 4, tetraploid root Fig 5, pentaploid root Fig 6, hexaploid root. Fig 7, $12n$ cell with 110 chromosomes from a chimaeral root of a hexaploid with $6n + 1$ chromosomes.

is illustrated in figs. 8–10 The two chromosome numbers are shown in figs 8 and 9, while a patch of cortical tissue, from the immediate neighbourhood of the diploid plate, is drawn in fig 10. Seven of the cells in this are sufficiently complete to be counted, though the nucleus of cell 4 was cut in half and has been reconstructed. The prophase nucleus of cell 6 is also mutilated to the extent of one and a half chromosomes, which are visible in the next section but which have not been drawn in. With this emendation, five cells (Nos 1, 2, 4, 5, and 6) show, to within one unit, either 18 or 36 chromatic bodies. The cells 3 and 7 show too few, but both were difficult to observe owing to the preponderance of nucleolar

surface in the views presented This degree of uncertainty is, however, immaterial for, in spite of it, it is possible, on the basis of prochromosomes alone, to draw the exact line of demarcation between the two types of



FIGS 8–10.—A chimaeral root of *B laevigata* × 11500 Fig. 8, diploid $2n = 18$. Fig 9, tetraploid in the same root, $4n = 36$ Fig 10, cortex at region of junction, the latter being shown by a thickened line.

tissue. The conclusion seems irresistible that a precise numerical correspondence does exist between the chromatic bodies of resting nuclei and the chromosome numbers, in ordinary polyploids of a species, whether these are separate plants or different parts of the same individual.

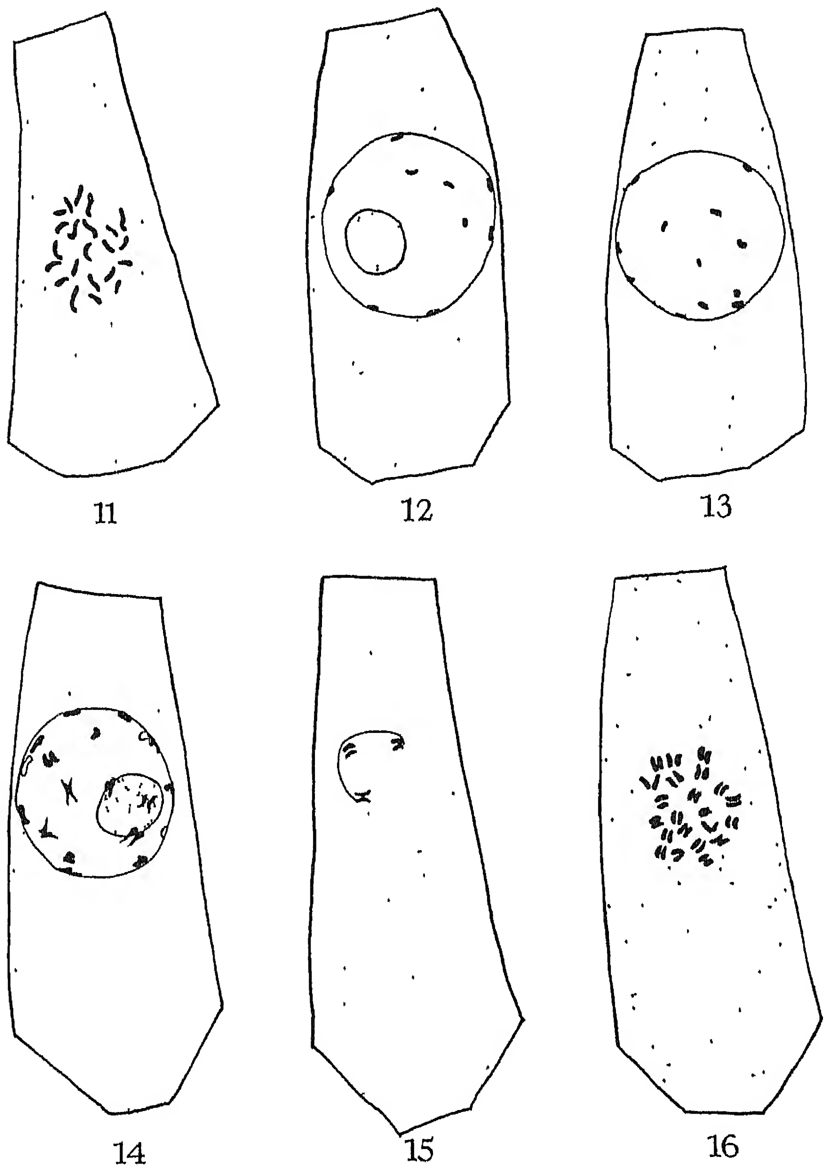
Evidence from chimaeras can, however, go farther than this, and a single preparation of a root of *Iberis semperflorens* L. is extraordinarily instructive. In this species the resting nuclei are of the same type as in *Biscutella*, fig 24, Plate 30, and the normal chromosome number is 22, fig. 25, Plate 30. In one preparation, however, a strip of tissue about 15 cells wide, in what is destined to be piliferous layer, contains not 22 single chromosomes but 22 pairs of chromosomes apparently in organic attachment, figs. 26 and 27, Plate 30 Seven metaphases of the kind figured occur in the space of 14 sections, and in the 16th section there is an anaphase with 44 chromosomes at each pole.

The appearances of the resting nuclei in this preparation are shown photographically in figs. 26, 28, and 29, Plate 30, and in greater detail in figs. 12–15 Figs 11–13 depict two superposed cells contained in three adjacent sections, from a normal part of the piliferous layer. An exactly comparable pair of cells from the abnormal region is represented in figs 14–16. The latter series superimposes in turn upon the cell marked x in fig. 28, Plate 30 It is evident that the normal cell contains the diploid number of prochromosomes, *i.e.*, $2n = 22$ In the abnormal tissue, on the other hand, although each cell possesses $4n$ chromosomes, as in the *Biscutella* chimaera, owing to their close association in pairs, $2n$ prochromosomes are still displayed These, however, show definite signs of doubleness, even when their actual size is small, and in certain cells, such as that marked y in fig. 28, Plate 30, and shown at a higher magnification in fig 29, this doubleness may be very marked. These particular cells may not be in rest but in prophase. They demonstrate, however, with absolute clearness that the occurrence of prochromosomal bodies depends not only upon the number of chromosomes present *but also upon their relative positions*

This double correlation cannot be explained away as an artifact. In the light of it the conclusion seems inescapable that in the Cruciferous nucleus the prochromosomes of fixed material do indeed represent structures in the living nucleus, in the sense put forward by Rosenberg.

Autonomy of Prochromosomes in Mitosis

Photographic demonstration of autonomy of chromatic structures throughout the mitotic cycle is presented in Plate 31. The material used is root meristem of both diploid and tetraploid *Biscutella* and the fixative is chrom-acetic formalin This reagent, though in some ways less convenient than the osmic fixative 2BE (La Cour) for the study of chromosome numbers (*see* Plate 30), is quite indispensable for the present purpose,



FIGS. 11–16—Cells from a chimaera of *Iberis semperflorens* L $\times$ 2000 Figs 11–13, two superposed cells from three adjoining sections of the normal piliferous layer, figs 12 and 13 being cut portions of the same cell $2n = 22$ and 22 prochromosomes. Figs. 14–16, two similar cells from the abnormal piliferous layer in the tier marked y in fig 28 Plate 30 $2n$ pairs of chromosomes and $2n$ double prochromosomes

since it alone causes nucleoli to stain differently from chromatin. With haematoxylin the nucleoli are pale yellow or at most grey instead of dense black That this is the truer picture is known from the similarly negative reaction of nucleoli to Feulgen's test (*cf* Doutreligne, etc).

Figs 31–38 in Plate 31 show a series of telophase stages from a single tetraploid root cut transversely The preceding stages of metaphase and anaphase are not included here from lack of space, but a portion of a L S showing both these phases in a diploid root is given in fig 45, Plate 32 The only special feature to remark in this is the characteristic flatness of the chromosome plates both on the equator and at the poles. The three cells in side view, figs 36, 37, and 38, show the transformation of the still flat plate of early telophase into the spherical nucleus of rest It is clear that this transformation involves the separation of the materials of the nucleolus and nuclear sap away from the chromatin The morphological expression of these events can be further studied in the series of polar views from the same preparation, figs 31–35. At late anaphase, fig 31, the chromosomes are densely crowded and apparently swollen, conditions which may, in this case, have been exaggerated by fixation; for this reason a comparable stage from a diploid specimen has been appended for comparison, fig 30. At early telophase, fig. 32, the individual chromosomes are at their least distinct. They never cease entirely to be discernible, but they lose their definite outlines and appear as if joined into a meshwork.* Many of these details are no doubt artifacts, for almost certainly the apparent meshwork is due to the precipitation, by the fixative, of nucleolar substance which is forming in the immediate neighbourhood of the chromosomes and which, in small quantities, may retain the stain , actually there is good reason to believe that this substance is at all times a liquid, and it behaves unmistakably as such in the later stages of telophase The middle stage of telophase is shown in figs. 33 and 34 in a pair of sister nuclei from two adjacent sections. The nuclear area has now increased markedly in size, and nucleolar substance in considerable quantity is present in irregular patches. In evident association with these patches are the chromosomes which are once more distinct from each other and have taken on the appearance of prochromosomes. Late telophase is marked by a strong development of nuclear sap and by a movement of the prochromosomes out to the surface of the nucleus, *see* figs. 35 and 37. At first the prochromosomes remain in contact with the nucleolar substance which becomes drawn out into threads in consequence. There is no evidence that this contact persists, the threads

* This stage is almost impossible to illustrate satisfactorily, and, in spite of considerable care, some of the web-like appearance of fig 32 has been lost.

disappear and the nucleolus coalesces into a single spherical mass or drop. Intermediate stages in these final processes are well seen in the figures last mentioned Movement of the prochromosomes has begun in fig 37, and the upper nucleus shows well the difference between the drawn-out condition of the nucleolus where it is still in contact with the chromosomes and the curvature of the surface on the lower side where contact has ceased (This figure also shows the beginning of the new cell wall) The lobing of the nucleolus in fig. 35 represents a stage in the running together of originally separate centres of material. Fig 38 displays the final state when mitosis is complete

It should perhaps be made clear that in all these telophase figures only an optical section can be photographed and not the entire nucleus. Thus out of 36 prochromosomes, fig 33 contains evidence of only 24 and fig. 34 of 31, the others being out of the focal plane That this is an optical effect due to the size of the whole structure and is not caused by any genuine lack of numerical correspondence is attested by a comparison with photographs of the corresponding stage in a diploid, figs. 39 and 40, Plate 31 Indications of 17 out of 18 prochromosomes appear in fig 39, and the whole complement of 18 is actually visible in fig 40.

Comparison of figs 33–34 and figs 39–40 brings out clearly another feature which may be pointed out in passing This is the similarity of configuration shown by sister nuclei By extending the comparison to an earlier stage, figs 41 and 42, it is obvious that the mutual resemblance of related nuclei at telophase is only slightly less than that at anaphase, and in both cases is the expression of their derivation from a common metaphase This of course is no new observation, for it has been known in other plants since the time of Strasburger It offers, however, in this instance, an additional confirmation of the fundamental truth of some of the fixation appearances here examined.

The subject of prophases will be passed over briefly, not from lack of interest but from the fact that polyploidy in itself has nothing new to contribute The abnormal chimaera of *Iberis* is in a different category in this respect, but unfortunately it cannot here be used. The original preparation was stained in gentian violet, in which condition it was photographed; but having twice faded to invisibility (in the course of eight years) and been restained it was finally brought into haematoxylin as a precaution against total loss While it is clear that such abnormal cells might throw considerable light on difficult details of mitosis, this cannot be sought in a thrice-stained slide

A full description of prophases in the Cruciferous type of nucleus involves, moreover, a very large measure of personal interpretation of

details, many of which are markedly affected by technique. In this respect neither of the fixatives employed has necessarily any superiority over those used by other investigators such as de Litardière, Doutreligne, etc. That many of the latter writer's observations can be repeated is shown by the photograph in fig. 43, Plate 31 The chromosomes here can be described as longitudinally split, since the dark central portion of each continues out into a pair of strands at each extremity (the ones most clearly in focus are marked by an arrow). Many such chromosomes also undoubtedly exhibit signs of a median constriction across the dark middle piece in the way described for *Impatiens* In other nuclei, however, fig. 44, the prophase chromosomes look very different, being long and slender and without trace of doubleness. This is possibly merely a later stage (as assumed by Doutreligne) which will be followed by a shortening and thickening of the now uniformly chromatic structures until the disappearance of nuclear sap heralds the onset of metaphase. The sequence of events is, however, obscured by the presence of a diversity of other nuclear appearances which I, personally, am unable satisfactorily to seriate. Some of these appearances are probably artificial distortions of identical stages in differently situated cells; but without more evidence this is difficult to assess. The present work will therefore not aim at either confirming or refuting Doutreligne's conclusions in detail. For the general point at issue it is sufficient to have shown that in prophase, no less than in telophase and throughout rest, chromatic bodies bearing a definite relation to chromosomes do exist.

THE NUCLEOLUS IN POLYPLOIDS—COMPARISON WITH ALLIUM

The information on nuclear structure obtainable from polyploid cells can be considerably extended by a comparison between the cases already considered and similar material from a different type of plant. It has long been known that the prochromosomal nucleus of the Cruciferae constitutes only one of two very different extreme forms. Rosenberg designated these as " Capsella Typus " and " Fritillaria Typus " respectively, and though other terminology* is often adopted the distinction, for

* *Note on nomenclature*—Sharp (1934) distinguishes the types of " slender and small chromosomes " from that of " large chromosomes " and the commonly used adjectives " prochromosomal " and " reticulate " refer to the same classification. Eichhorn has recently advocated the use of " homogeneous " to contrast with " reticulate." The word homogeneous, however, merely seems to denote the structureless nature of the nuclear sap, and it is certainly a misnomer as applied to the whole nucleus In the present work two pairs of terms will be used, the older words " prochromosomal " and " reticulate " and two other adjectives descriptive of the new data and explained in the text, namely, " vesicular " and " solid " (*see* pp. 534 and 537)

descriptive purposes, is still often found to be a convenient one (*e.g.*, Sharp, 1934). The Capsella type is that of *Biscutella* and *Iberis* and in it, as already shown, the bulk of the stainable chromatin is in the superficially placed prochromosomes The Fritillaria type, on the other hand, possesses a "reticulum," *i e*, an appearance after fixation as of a uniformly chromatic meshwork filling the whole nucleus except for the space occupied by one or more nucleoli In this reticulum some denser aggregates of chromatin, often termed chromocentres (*cf.* Heitz, 1932, etc), may also be incorporated; but these are relatively inconspicuous and not necessarily superficial in position *Vicia*, *Osmunda*, many Liliaceae, etc , may be quoted as examples, and all of these possess chromosomes of large size This correlation with chromosome size is the reason why the reticulate nucleus figures predominantly in the literature and why many of the species concerned have been selected repeatedly for cytological study. Comparisons are rendered easier by this fact, for several aspects of the behaviour of large chromosomes may be assumed to have been already correctly described On the other hand, several fundamental characteristics have escaped observation, in spite of many comparative works published since 1904, *e g*., Lundegård (1913), de Smet (1914), de Litardière (1921), Sharp (1934), Eichhorn (1935). This is in part due to a tendency of the investigators to concentrate closely on details, *e.g.*, of chromatin distribution, rather than on general features. But in any case the increased technical scope made possible by the application of polyploidy and photography is its own justification. This section will therefore embody a comparison, on general lines, between *Biscutella* and a single chimaeral specimen of *Allium ursinum*

Characters of great value for comparative purposes are afforded by the behaviour of nucleoli A glance at fig 1 shows immediately that in *Biscutella*, no matter how many chromosomes may be present in a cell, the nucleolus at rest is always single It varies in size with the degree of polyploidy, a fact which is consistent with the participation of all the chromosomes in the initial deposition of the substance (*cf.* Plate 31). But an invariable feature of telophase is the amalgamation of the nucleolar substance from all parts of the nucleus into one central structure In *Allium*, on the other hand, the nucleoli are at all times multiple They are, moreover, related not in size but in number to the chromosomes present.

Detailed elaboration of the above statements for *Allium* is unnecessary, since they are in accord with accounts of some other cases of polyploidy already published, *e.g.*, Yeates (1925) on *Tmesipteris*, de Mol (1928) on *Hyacinthus*, Derman (1933) on *Petunia.* Nevertheless, photographic demonstration is appended Figs 46 and 47, Plate 32, show metaphase

chromosomes from two parts of a chimaeral root at a magnification of $\times$ 1000 ($2n = 14$, $4n = 28$) Figs. 48 and 49 are general views of the two kinds of tissue at half the magnification, and figs 55 and 56, Plate 32, show optical sections of two isolated nuclei in greater detail Both are telophases, but that of fig 55 (the tetraploid) is at a somewhat later stage than that of fig 56 In the latter, 4 nucleoli are visible, and this is the maximum number possessed by a diploid cell In the tetraploid tissue the maximum number is 8, though the photograph only shows six separate bodies. Two of the largest of these are, however, bilobed, which indicates that they each represent two which have fused laterally Such lateral fusion occurs to a limited extent in both diploid and tetraploid and will be referred to again

The difference in the appearance of the nucleoli in *Biscutella* and *Allium* is due in part to a difference in their mode of origin It is now generally accepted (*see* summary in Sharp, p 118) that where chromosomes are large the nucleolar substance at telophase does not accumulate promiscuously in contact with all of them (as in *Biscutella*, *Impatiens*, Doutrelıgne, etc). Instead it appears to form selectively in the neighbourhood of only certain portions of specific chromosomes These chromosomes are often morphologically distinguishable (*e g* , Levan, 1935, for a description of *Allium*), and, since they are present in a definite number, the initiation of a fixed number of nucleoli is explained (Heitz, 1931 and 1932) The symmetrical arrangement of nucleoli in sister cells is also regarded as an expression of the same fact (*cf.* the top right-hand telophase of fig 51). These ideas are, however, of only minor importance in the present connexion, as will be immediately evident They will therefore not be pursued

It is clear that the mode of origin of nucleoli does not in itself determine their subsequent behaviour Of far greater importance is the *physical state of the nucleus as a whole* This is also the main subject of the present enquiry It will be recalled that even in *Biscutella* the nucleolus, though single at rest, was frequently not so at an earlier stage, *e g* , figs 33–35, Plate 31. It only became so by the flowing together of originally separate patches of material as contact with the chromosomes was loosened. The telophasic movement of prochromosomes out to the nuclear surface is thus an integral part of the process of nucleolar fusion or, in other words, the single nucleolus is an expression of a *vesicular* nuclear construction. In *Allium*, on the other hand, the nucleoli do not coalesce, owing to the fact that at no time are they free to do so. The nucleolar substance is still a liquid, and adjacent nucleoli will flow together if they arise sufficiently near each other to come into contact They remain

permanently separate, however, if contact is prevented by the presence of intervening substances The multiple nucleolus is therefore an indication that the nucleus of *Allium* is not a vesicular structure but relatively *solid*

The meaning of the term "solid" as applied to the nucleus of *Allium* can be more precisely defined after reference to the mitotic cycle in this plant has been made For this purpose general views of meristematic cells in an L S of a normal root are given in figs 50 and 51 at a magnification of × 1000 Greater detail of certain stages from the chimaeral root is given at a magnification of × 2000, in figs 52–57, Plate 32, and, to the same scale, a portion of an L S of a root of diploid *Biscutella* is added for comparison, fig 45

It can now be shown that *Allium* differs from *Biscutella* in four salient features (1) size of its chromosomes, (2) absence of a flat plate both at metaphase and at anaphase; (3) absence of chromosome movement at telophase; and (4) a proportional difference in the amounts of the various nuclear components, *i.e.*, chromatin, nucleoli, and sap These points will be dealt with briefly in turn

(1) The question of chromosome size need not be dwelt on, since it has already been partially discussed (p. 533) The average dimensions of a chromosome in *Allium* are 13 $\mu \times 1$ μ,* the width being measured in the immediate neighbourhood of the spindle attachment constriction. In *Biscutella* average measurements are 1 8 $\mu \times$ 0 4 μ These figures may be verified in the photographs of fig 18, Plate 30, and fig 46, Plate 32, it being remembered that they are to a scale of × 2000 and × 1000 respectively.

(2) The absence of a flat plate is well seen in the metaphase of fig. 50 and the anaphase of fig. 51. Both are in striking contrast to the same stages in fig 45 The difference is probably in part merely a mechanical effect of the length of the chromosomes in relation to the size of the cell Whatever the cause, however, the morphological consequences on the procedure of telophase are significant This will be evident from the next paragraph.

(3) The absence of chromosome movement at telophase in *Allium* is a feature of considerable importance which requires and deserves fuller demonstration. This is obtainable from the large-scale photographs in the lower half of Plate 32. In these it can be shown with certainty that the bunched condition of the chromosomes of anaphase persists unchanged from the end of one mitosis until the beginning of the next. At telophase

* A recent record by Levan (1935) gives the size of the largest chromosome in *A ursinum* as 14 $\mu \times 1$ μ These figures are therefore confirmed

the chromosomes retain, until a late stage, their visible identity as V-shaped structures with long arms In fig. 57, which is a surface view of a diploid nucleus in middle telophase, the bent portions of the V's can be seen in relation to the pole (the clearest one is marked by an arrow) In fig 56, which is the same nucleus at a lower focus, only the closely crowded arms are encountered These practically fill the nucleus (except for the space occupied by nucleoli), and since they lie almost parallel to each other they appear roughly circular in optical section As telophase advances, *cf* the tetraploid nucleus of fig 55, the sectional area of the chromosome arms increases and the spaces between them diminish until at rest their outlines are indiscernible. At prophase, however, they reappear *in the same relative positions.* Figs 52 and 54 are optical sections of early and late prophases in tetraploid cells, and fig. 53 is a surface view at the polar end of the same nucleus as fig. 54. If fig. 53 is compared with fig 57 it is evident that the same arrangement of V-shaped chromosomes converging to a pole is displayed by both. The anaphase configuration thus persists unaltered from telophase to prophase. No mutual displacement of chromosomes can therefore be involved in the attainment of the resting state.

Conclusions of such importance follow from this fact that it is worth interrupting the presentation of evidence to consider them. The absence of telophase movement means that the "intervening substances" filling the space between and round the nucleoli are nothing other than the chromosomes themselves, transformed but not displaced. Another corollary is also implied. The fact that the transformed chromosomes are able not only to maintain their own positions but also to enforce a similar stability upon the nucleoli proves that the telophase changes cannot involve the complete loss of mechanical rigidity It is inconceivable that such fixity of spacial arrangement could be maintained against external forces of deformation, if the material basis of the resting nucleus were either structureless or liquid That such external forces are not negligible is proved by the extreme changes of shape imposed on the whole nucleus in the cells of the developing stele. Here the nuclei may be compressed to half their previous width and elongated to more than 12 times their previous length, but the spacial separation of nucleoli is still maintained Even at rest, therefore, some of the properties of elastic solids must be attributed to the chromosomes of *Allium*

(4) The final evidence to be considered is that of the relative proportions of the nuclear components in the two plants The calculations of these are given in full in the Appendix, and only the more general results will be mentioned Briefly they amount to this in relation to the diploid

Biscutella the average nucleus of *Allium* has eight times the total volume, five times the volume of nucleoli, but 36 times as much chromatin at metaphase The first two of these ratios are not in themselves significant. The total nuclear volumes are of the same order of magnitude and could have been still more alike had a higher polyploid of *Biscutella* been selected The extent to which the nucleus is occupied by nucleolus is also similar in each, for the difference between 5 and 8 may merely reflect the difficulty of fully evaluating the aggregate total in *Allium* It is the association of these figures with the third that marks the comparison Bulk for bulk the nucleus of *Allium* has incorporated over four times as much chromatin as has that of *Biscutella* This fact supplements, but is not synonymous with, the observations already made on the individual dimensions of the chromosomes

The question of nuclear sap remains to be mentioned. In *Biscutella* this fills the cavity between the nucleolus and the prochromosome layer, but in *Allium* it is extremely doubtful if any at all is present as a free liquid before the onset of prophase Its appearance at this stage is marked by a considerable increase in the diameter of the whole nucleus and by the loosening of the previously compact mass of chromosomes (*compare* figs 52 and 53, Plate 32)

This absence of free sap at telophase has in its turn an important corollary. It means that the increase in nuclear volume which invariably accompanies the transition from metaphase to rest is, in the case of *Allium*, not external but *internal* to the chromosomes themselves The magnitude of the change has been calculated in the Appendix Here the volume of the resting nucleus, after subtraction of the amount occupied by nucleoli, has been compared with the total volume of the metaphase chromosomes The ratio is approximately 5 · 1 Each chromosome must therefore swell to five times its previous size during telophase.

This is as far as the present analysis can profitably be carried

CONCLUSIONS

The two types of nuclei may now be more precisely defined The "solid" nucleus is a structure devoid of free sap and composed almost entirely of the greatly enlarged bodies of chromosomes closely appressed. A characteristic number of nucleoli is intercalated between them and the relative positions of both nucleoli and chromosomes are determined at anaphase. The telophase changes, except for the secretion of nucleoli, are largely internal to the chromosomes, and the attainment of a spherical form by the final nucleus does not involve chromosome movement.

The resulting structure is relatively rich in chromatin and must possess an appreciable mechanical rigidity. The nucleoli, in the early stages at least, are liquid

The "prochromosomal" nucleus, on the other hand, is a vesicular structure in which a relatively enormous nuclear volume is attained by the accumulation of sap outside the relatively small chromosomes. Extensive movements of these are involved in the conversion of the flat anaphase plate into the spherical condition of rest. These movements are also connected with the fusion of all the nucleolar substance into a single central body The resulting nucleus is relatively poor in chromatin The interior is presumably fluid, since it consists only of sap and nucleolus. The periphery is presumably more rigid, for it contains the whole of the stainable chromatin and all that is visible of the bodies of the chromosomes, namely the prochromosomes

Discussion

It is suggested that the above descriptions of some resting nuclei in plants are both truer and more useful than either of the generalized statements quoted in the introduction. All the facts presented seem to be incompatible with an "essential homogeneity of the interkinetic nucleus" in the sense upheld by Gray (1931, p 122). On the other hand, the limitations of the description, "The reticulum is composed of threads" (Sharp, 1934, p 54), is also apparent Since both statements are of importance in current cytology, it may be of service to discuss them briefly in the light of the new facts. It will be convenient to treat first the opinion of Sharp

Criticism of the term "reticulum" must be chiefly negative. It may have been observed that the precise details of chromatin distribution have figured very little in the present work. This is, in the first place, because they are of only subordinate importance relatively to the more general features considered. The reticulum of a solid nucleus may or may not contain chromatic bodies, "chromocentres" (*cf* p 533) bearing a certain resemblance to prochromosomes, its solidity is still the dominant feature Conversely, it is the peripheral position of the chromatin, not the mere presence of prochromosomes, which distinguishes the vesicle. Many animal cells (*e.g.*, eggs of *Echinus* and *Asterias*, *see* below) appear to be vesicular without being prochromosomal The superficial chromatin in these could be described as "reticulate," but unless the term is qualified by an explanation that it applies only to the nuclear surface its use can be most misleading

The details also of the reticular conception may be criticized on a second ground They involve the interpretation of the *internal* structure of chromosomes, a difficult matter at all times, but in the resting nucleus the difficulty is enhanced by the swollen state of the structures. The telophase enlargement calculated for *Allium* amounts to a doubling of the diameter of each chromosome To describe such a swollen chromosome as a " *delicate* thready structure " is strongly suggestive of a contradiction in terms The paradox cannot be resolved without further evidence, but it does not diminish by closer inspection of the conditions. The physico-chemical basis of telophase enlargement is unknown, but the behaviour of nuclear sap suggests that one factor may be nothing other than transference of water If this should be so and if the degree of hydration of a colloidal system should affect in any way the pattern of its precipitation by a fixative, then clearly the utmost caution is necessary. The truth, in details, of the appearance of the same chromosome fixed at prophase and at telophase may be very different This is the reason why no attempt has been made to interpret the present evidence in greater detail or in terms of chromosome structure

Before leaving this subject, one other conclusion relevant to morphological cytology in general may be stressed. It arises from the contrast between a prochromosome and a reticulum. The differences described between large and small chromosomes appear to be not merely quantitative but qualitative A small chromosome is not merely a large chromosome in miniature and, conversely, the structure of large chromosomes is not necessarily more clearly revealed by virtue of their size This fact has frequently been overlooked It should, however, be specially borne in mind when any attempt is made to generalize from statements based on specific observations. Thus the universality of spiral structure, to choose an example at random, is still unproved, even though its reality in certain instances (*e.g* , at meiosis in *Tradescantia*, etc , Nebel (1932), Kuwada (1932), Sax (1934), etc.) appears to be authentic

With regard to experimental cytology it may be remarked that the attribution of structure to nuclei, and structure of more than one general type, is the direct result of the morphological data presented Nevertheless this evidence, strong as it is, does not stand alone It can be supplemented by the results of direct experiment. The application of micro-dissection methods to plant cells is still only in its initial stages, but, few as are the records, a paper by Chambers and Martens (1932) is closely relevant to the point at issue The nucleus of *Tradescantia* (a plant known to be of the *Fritillaria* type) was found to be relatively indifferent to the entry of a micro-needle, although the lethal effect of withdrawal of

the needle caused extensive optical changes. This behaviour was contrasted with "un matériel animal varié et aussi sur *l'Allium*, l'un de nous a montré, ici encore, que la piqûre du noyau—souvent même la simple piqûre de la cellule—provoquait une liquéfaction ou une coagulation nucléaire définitive, tandis que dans d'autres cas (oeuf d'*Asterias*) il était possible de *déplacer la pointe de l'aiguille a l'intérieur du noyau, sans apparente altération*" (*loc. cit*, p. 139; the italics are mine). Without offering any explanation for the apparently exceptional resistivity of *Tradescantia*, it may be pointed out that the general comparison here made is just what would be expected to occur in the behaviour of vesicular as opposed to non-vesicular nuclei One further piece of experimental evidence may be quoted The well-known observation of Gray (1931, p 125) on the response of the nucleolus to gravity in the egg of *Echinus* is sufficient indication that the nucleus here is a vesicle This may be contrasted with the experience of Ortiz Picon (1931), who was unable to disturb the position of the nucleoli of *Allium* even by the application of considerable centrifugal force * This is strong confirmation of the inferred solidity

Perhaps enough has now been said to show that collation of morphological facts with "fundamental structure of living nuclei" is not impossible The limitations of morphology in certain directions are not denied, they have rather been stressed. Recognition of the extent to which morphological information is reliable, on the other hand, is much to be desired Many apparently contradictory observations on living cells become interpretable in simple terms (as in the preceding paragraph), and errors caused by confusion of facts from different types of nuclei may be avoided

Finally a word may be added on the subject of photography. It has been felt very strongly throughout this investigation that the camera far excels every other means of illustration in the cases where it is applicable. Not every subject can be photographed, it is, for instance, impossible to include in one focus all the bodies on the surface of a sphere. But where optical limitations are not insuperable a successful photograph is often the only means of providing ocular demonstration of evidence without

[*Footnote added in proof, October* 3, 1935—This reference to "intensa centrifugación" is derived from Tischler's quotation of Ortiz Picon (*loc. cit* p. 107), I have unfortunately been unable to obtain the original work Another paper of much interest has, however, appeared since going to press (Beams and King, 'Proc Roy Soc' B, vol 118, p 264 1935) Here a force of 400,000 g seems to have had surprisingly little effect on the root nuclei of the bean *Phaseolus* It is hoped to pursue this subject further]

involving either diagrammatic simplification or a strong element of personal interpretation by the draughtsman. It has also been felt that the very limitations of the camera, its inability to record more than one focal level at a time, can be a valuable asset. It may serve to concentrate the attention on an unusual view in a way which can be an appreciable aid to observation.

Summary

By combining evidence from polyploid individuals and polyploid chimaeras of various kinds in two species of the Cruciferae, *Biscutella laevigata* and *Iberis semperflorens*, it has been shown that the prochromosomes of meristematic cells correspond in number *and in position* with the chromosomes This is considered to be conclusive proof of the reality of some of the morphological deductions from fixed material.

By observation of the whole mitotic cycle in diploid and tetraploid roots of *B. laevigata*, the correspondence of chromosomes with prochromosomes at all stages has been confirmed. The facts have then been used to elucidate some fundamental attributes of a vesicular type of resting nucleus. Three characteristic features of telophase have been shown to be involved (i) the accumulation of sap *outside* the small chromosomes; (ii) extensive *movements* of the telophase chromosomes away from the nucleolus and out to the nuclear surface; (iii) fusion of all the nucleolar substance into a single central body

The nucleoli of a chimaera of *Allium ursinum* have been shown to contrast with those of *Biscutella*, by being always multiple and by showing a *numerical* increase in the polyploid tissue This has been collated with the fact that *Allium* possesses large chromosomes and a "reticulate" type of resting nucleus. This behaviour of the nucleoli is regarded as proof of the maintenance of some mechanical rigidity by the chromosomes both during telophase and at rest

Observation of mitosis in *Allium* has been used to elucidate some fundamental attributes of a "reticulate" type of resting nucleus This has been shown to be a relatively solid structure rich in chromatin and devoid of free sap until prophase *No* chromosome movements are involved in telophase, and increase in the total volume during telophase is largely *internal*, not external, to the chromosomes

Photographic demonstration of all the cytological facts is provided and the importance of this is stressed.

APPENDIX

Calculations of Sizes of Nuclear Components in *Biscutella* and *Allium*

Unit of Measurement—Most values are measured in terms of millimetres at a magnification of $\times$ 2000 Where desirable the absolute sizes in μ are also given

I—*Nuclear Dimensions*

Allium—The outlines of 10 diploid nuclei in the chimaeral root were traced with a camera lucida at a level adjusted to give the required magnification Diameters were then measured on the drawings in two directions at right angles The values varied from 17 mm to 27 mm and the average was 23 6 mm

Therefore mean radius of nucleus = 11·8 mm = approximately 6 μ.

Biscutella—The same method was adopted except that an L.S. was used instead of a T S. This was to allow for the flattening of nuclei often shown in Cruciferous roots where the cells of the active meristem may be very short. By using only nuclei, which appeared roughly circular in side view, diameters varied from 11 mm to 13 5 mm with the average at 12·3 mm.

Therefore mean nuclear radius = 6·15 mm approximately 3 μ.

Thus in relative terms *Biscutella* has half the nuclear radius of *Allium*. It therefore possesses one-eighth of the nuclear volume.

II—*Nucelolar Dimensions*

Nucleoli were measured in each case from the same 10 nuclei previously used, and the measurements were again made on the drawings.

Allium—The multiplicity and irregularity of shape of the nucleoli here makes accuracy impossible For an approximate estimate, each nucleolus was treated separately and its radius calculated from two measurements at right angles The cube of each radius was then calculated and the effective value of r for a whole nucleus was obtained by adding them all together The full figures for the 10 nuclei are:

(1) Diameters = 11 and 6, therefore radii = 5 5 and 3
Therefore $r^3 = 166 + 27 = 193$.

(2) Diameters = 11 and 2 5 and 3, therefore radii = 5·5 and 1·25 and 1 5
Therefore $r^3 = 166 + 3 + 2 = 171$.

(3) Diameters = 8·8 and 5 and 6, therefore radii = 4·4 and 2·5 and 3.

Therefore r^3 = 85 + 16 + 27 = 128.

(4) Diameters = 8·5 and 6·2 and 5·5, therefore radii = 4·25 and 3·1 and 2·75.

Therefore r^3 = 77 + 30 + 21 = 128.

(5) Diameters = 6·2 and 2·5 and 8·5, therefore radii = 3·1 and 1·25 and 4·25.

Therefore r^3 = 30 + 2 + 77 = 109.

(6) Diameters = 4·5 and 4·2 and 6·8, therefore radii = 2·25 and 2·1 and 3·4

Therefore r^3 = 11 + 9 + 40 = 60.

(7) Diameters = 5 and 4·5 and 7·5, therefore radii = 2·5 and 2·25 and 3·75

Therefore r^3 = 16 + 11 + 53 = 80

(8) Diameters = 6·5 and 5 and 3·6 and 8·5, therefore radii = 3·25 and 3 and 1·8 and 4·25.

Therefore r^3 = 35 + 27 + 6 + 77 = 145.

(9) Diameters = 7·8 and 7·5 and 4, therefore radii = 3·9 and 3·75 and 2

Therefore r^3 = 59 + 53 + 8 = 120.

(10) Diameters = 8·5 and 7·5 and 3·5, therefore radii = 4·25 and 3·75 and 1·75.

Therefore r^3 = 77 + 53 + 3 = 133

Thus the average value for r^3 = 127.

Therefore average nucleolar volume ($4/3\pi r^3$) = 532 mm^3 This figure, from the nature of the difficulties, is probably too low.

Biscutella—The single spherical nucleolus here presented less difficulty. Measurements of diameters ranged from 5·5 to 7 mm, with the great majority at 6 mm

Therefore average radius = 3 mm = 1·5 μ.

Therefore r^3 = 27

Therefore average nucleolar volume = 113 mm^3.

Thus in relative terms *Biscutella* possesses one-fifth of the nucleolar volume of *Allium*.

III—*Chromosome Sizes*

Allium—Measurements of those chromosomes most completely in focus in the photograph, fig 46, Plate 32, gave approximate dimensions of $13\mu \times 1\mu$, the length being the sum of the two arms of each chromosome (usually about $5\mu + 8\mu$) and the width that of the immediate neighbourhood of the attachment constriction In terms of millimetres $\times$ 2000 the approximate radius = 1 mm, approximate length 26 mm

Therefore approximate volume of one chromosome = 26π = $81 \cdot 6$ mm^3.

Therefore total volume of chromosomes per nucleus = $81 \cdot 6 \times 14$ = 1143 mm^3

Biscutella—Measurements of a number of chromosomes from the photographs of the various polyploids gave average dimensions of 3 6 mm $\times$ *ca.* 8 mm (= 1 $8\mu \times 0{\cdot}4\mu$)

Therefore approximate radius = $0 \cdot 4$ mm

Therefore approximate volume of one chromosome = $\pi \times 0 \cdot 16 \times 3 \cdot 6 = 1 \cdot 8$ mm^3.

Therefore volume of chromosomes per diploid nucleus = $1 \cdot 8 \times 18$ = 32 mm^3

Thus in relative terms *Biscutella* has 1/36th of the chromatin possessed by a nucleus of *Allium*.

IV—*Calculation of Chromosome Swelling in* Allium

From the dimensions given in I it follows that the total volume of a nucleus of *Allium* = $4/3\pi \times 11 \cdot 8^3 = 4 \cdot 19 \times 1643 = 6884$ mm^3.

From II it is known that the approximate volume occupied by nucleoli = 532 mm^3.

Therefore the volume occupied by chromosomes in the resting nucleus

$$= 6884 - 532 = 6352 \text{ mm}^3.$$

From III it is known that the volume of the chromosomes at metaphase = 1143 mm^3.

Therefore the ratio of the chromosome volumes at metaphase and at rest is roughly as 1 . 5. Without change of length this would almost be achieved by a doubling of their diameters during telophase.

References

Chambers, R , and Martens, P (1932). 'La Cellule,' vol. **41**, p 131.
Dermen, H (1933). 'J Arnold Arboretum,' vol. **14**, p. 282
Doutreligne, J. (1933). 'La Cellule,' vol **42**, p. 31.

Eichhorn, A (1932) 'Rev. gen. Bot,' vol **44**, p. 260.
—— (1935). 'Rev Cyt Cytophys Veg, Paris,' vol. **1**, p 150
Gray, J (1931) "A Text-book of Experimental Cytology," Cambridge
Gregoire, V. (1907) 'Ann Soc zool malac Belg,' vol. **42**, p. 267
—— (1931) 'Bull. Acad Belg, Cl. Sci,' vol. **42**, p 1435
Heitz, E (1929) 'Ber deuts. bot Ges,' vol **47**, p. 274
—— (1931) 'Planta,' vol **15**, pp 495–505
—— (1932) 'Planta,' vol **18**, pp 572–636
Kuwada, Y (1932) 'Bot Mag Tokyo,' vol **46**, p 257
Laibach, F (1907) 'Beih bot Zbl,' vol **22**, pt. I, p. 191.
Levan, A (1935) 'Hereditas,' vol **20**, p 269
de Litardière, R. (1921) 'La Cellule,' vol. **31**, p 255
Lundegârd, H (1913) 'Arch Zellforsch,' vol **9**, p 205
Malte, M O (1908) 'Bot Notiser,' p 75
Manton, I (1932) 'Ann Bot,' vol **46**, p 509
—— (1934) 'Z indukt Abstamm-u VererbLehre,' vol **67**, p 41
de Mol, W. E (1928). 'La Cellule,' vol **38**, p 7.
Nebel, B R (1932). 'Z Zellforsch.,' vol **16**, p. 252
Ortiz Picon, J M. (1931) 'Bol. Soc Espan. Hist. Nat. Madrid,' vol **31**, p. 611
Rosenberg, O (1904) 'Flora,' vol. **93**, p 251
—— (1909). 'Svensk bot. Tidskrift,' vol **3**, p. 163
Sax, K., and Humphrey L (1934) 'Bot. Gaz,' vol **96**, p. 353
Sharp, L W. (1934) "An Introduction to Cytology," New York, 3rd ed
de Smet, E (1914) 'La Cellule,' vol **29**, p. 335
Tischler, G (1934). "Allgemeine Pflanzen karyologie," Berlin, 2nd ed.
Yeates, J. S. (1925) 'Proc. Roy Soc,' B, vol **98**, p 227

Description of Plates

Plate 30

Fig 17—Monoploid ("haploid") *Biscutella laevigata* L $n = 9$. The second meiotic division in a pollen mother cell of a diploid plant (subsp. *alsatica*) × 2000

Fig. 18—Diploid *B laevigata* $2n = 18$, from a root of subsp *alsatica*, fixed in 2BE, stained in gentian violet × 2000

Fig. 19—Triploid *B laevigata*, $3n = 27$, from a root of a hybrid between subsp. *alsatica* and subsp *longifolia*, fixed in 2BE, stained in haematoxylin. × 2000.

Fig 20—Tetraploid *B. laevigata*, $4n = 36$, from a root of subsp *longifolia* fixed in 2BE, stained in gentian violet × 2000

Fig 21—Pentaploid *B laevigata*, $5n = 45$, from a root of a hybrid between the Spanish hexaploid and subsp *longifolia*, fixed in 2BE, stained in gentian violet. × 2000

Fig 22—Hexaploid *B laevigata*, $6n = 54$, from a root of a Spanish plant, fixed in 2BE, stained in haematoxylin × 2000

Fig. 23—Resting cell of the $12n$ chimaeral tissue from a Spanish hexaploid, drawn in fig. 7, fixed in 2BE, stained in gentian violet × 2000

FIG 24—*Iberis semperflorens* L, a piece of the normal root meristem showing resting nuclei and chromosomes, fixed in chrom-acetic formalin, stained in gentian violet × 920

FIG 25—The chromosome plate of fig 24 enlarged $2n = 22$ × 2000.

FIG 26—*Iberis semperflorens*, a piece of the abnormal region of the same root × 920

FIG 27—The chromosome plate of fig 26 enlarged to show 22 pairs of chromosomes × 2000

FIG 28—Another section of the abnormal region of *Iberis* showing resting cells of which x is enlarged in fig 29 and y shows the position of the tier drawn in fig. 3.

FIG 29—The portion marked x in fig 28 enlarged to × 2000 Double prochromosomes are visible

PLATE 31

The mitotic cycle in the root of *Biscutella laevigata* fixed in chrom-acetic formalin, stained in haematoxylin and all × 2000

FIG 30—Late anaphase, polar view, diploid root

FIG 31—Late anaphase, polar view, tetraploid root

FIG 32—Early telophase, polar view, from the same tetraploid root

FIGS 33 and 34—Middle telophase, polar view of sister cells in adjoining sections, from the same tetraploid root Note the similarity of configuration and the relation of the nucleolar substance to the chromosomes.

FIG. 35—Late telophase, polar view, from the same tetraploid root, to show the separation of the prochromosomes from the nucleolus and the flowing together of all the nucleolar substance into one body.

FIG 36—Side view of early telophase, from the same tetraploid root.

FIG. 37—Side view of fairly late telophase, from the same tetraploid root. The process of separation of the chromosomes from the nucleolar material is well seen in the upper nucleus. Between the nuclei is the new cell wall.

FIG 38—Side view of two daughter cells at the end of mitosis, from the same tetraploid root. The peripherally situated prochromosomes and the now spherical nucleolus are well seen Both nuclei are cut and the sap-filled perinucleolar cavity is exposed

FIGS 39 and 40—Middle telophase in a diploid root, sister cells comparable to figs 33 and 34 Fig 39 shows all 18 prochromosomes, fig 40 only 17 of them.

FIGS 41 and 42—Anaphase in the same diploid root, two plates in the same cell to show similarity of configuration

FIG 43—Prophase in a tetraploid root, the chromosomes appearing split at their ends

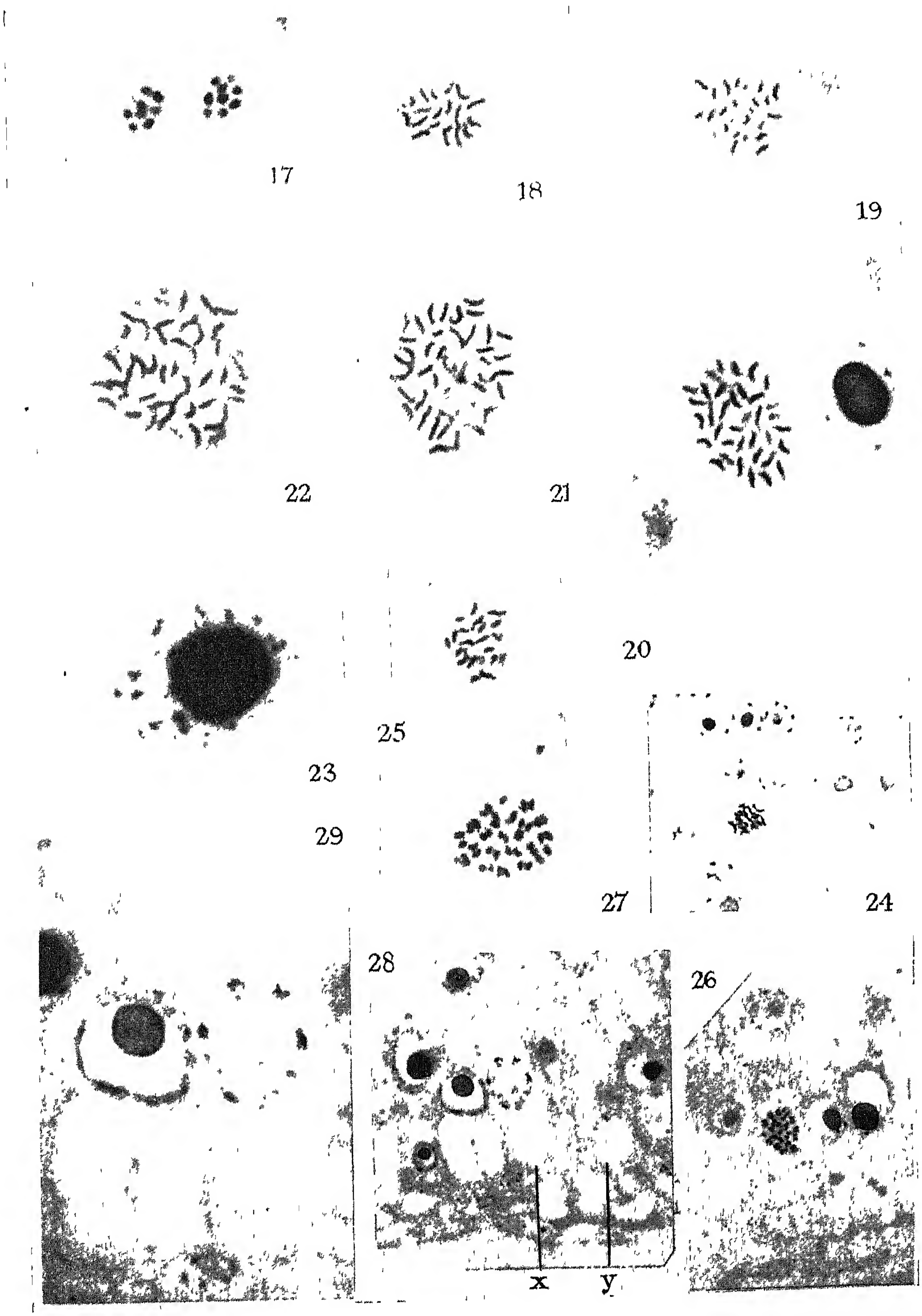
FIG 44—Prophase in a tetraploid root, chromosomes long and thin, not visibly double (*see* text, p 532)

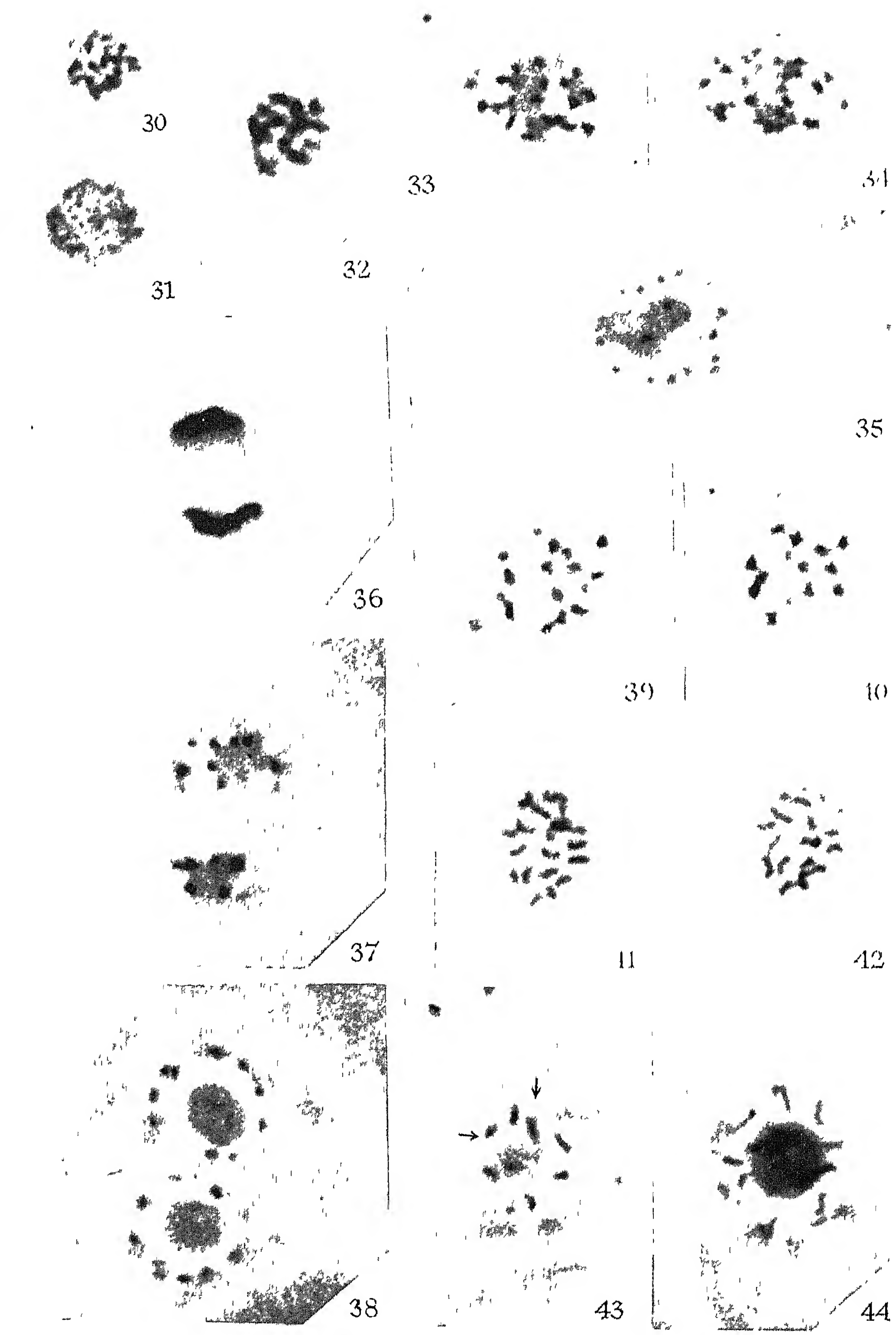
PLATE 32

FIG 45—*B laevigata*, portion of an L S of a diploid root showing nuclei at rest, metaphase and anaphase × 2000

FIG 46—*Allium ursinum* L chromosomes of diploid tissue of a root fixed in 2BE, stained in haematoxylin $2n = 14$ × 1000

FIG 47—Chromosomes in tetraploid tissue from the same root × 1000.


17
18
19
22
21
20
23
25
29
27
24
28
26
x
y


30
31
32
33
34
35
36
37
38
39
40
41
42
43
44

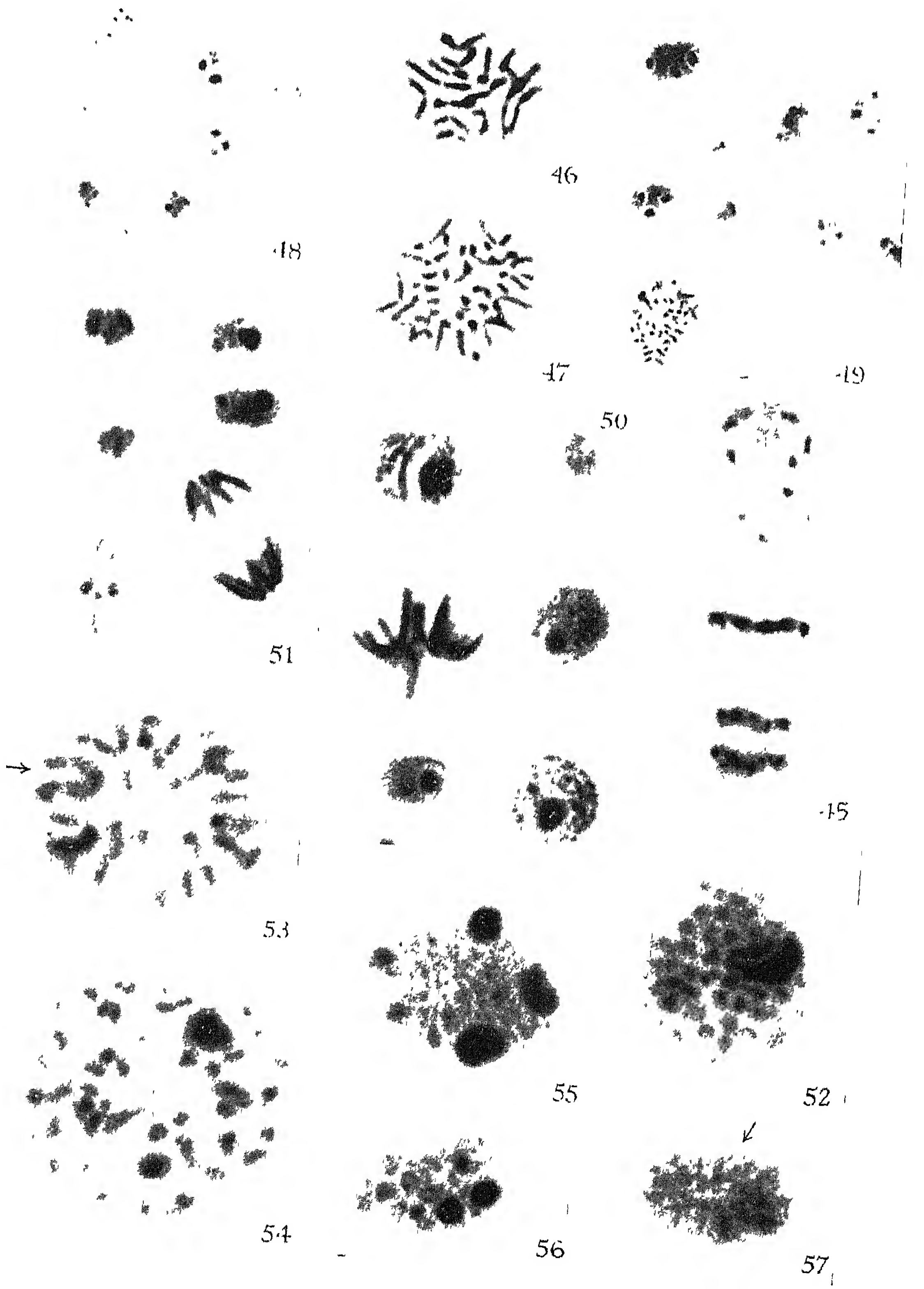

46
48
47
49
50
51
45
53
55
52
54
56
57

FIG. 48—General view of diploid tissue in the same root to show nucleoli in resting cells × 500

FIG. 49—General view of tetraploid tissue in the same root to show increase in number of nucleoli. × 500

FIG. 50—General view of mitosis in a normal root of diploid *Allium ursinum* cut longitudinally, fixed in 2BE, stained in haematoxylin The stages contained are rest, prophase, and metaphase × 1000

FIG. 51—Another part of the same showing anaphase, early and late telophase, and a cut metaphase × 1000

FIG. 52—Optical section of early prophase in a tetraploid cell of the chimaera of *Allium* × 2000

FIGS. 53 and 54—Late prophase in a tetraploid cell of the same, two views of one nucleus. × 2000 In fig. 53, the surface view of the polar end, the bent portions of V-shaped chromosomes radiate from a clear central area. Each chromosome is split and spirally twisted In fig. 54, an optical section through the middle of the nucleus, the effect of the appearance of nuclear sap is evident (*compare* with fig. 52).

FIG. 55—Late telophase in a tetraploid cell of the same root, to show the number of nucleoli. Six separate nucleoli are visible, of which two are bilobed and the result of fusion. The outlines of the optical sections of the chromosome arms are just discernible in the body of the nucleus (*compare* fig. 56). × 2000

FIG. 56—Middle telophase in a diploid cell of the same root, to show the number of nucleoli The whole complement of 4 is visible, and the rest of the nucleus is filled with chromosome arms appearing in optical section × 2000.

FIG. 57—Surface view of the same nucleus as fig. 56 The anaphase positions of the chromosomes are still detectable by the bent portions of V's which converge to a central clear region which marks the pole (*compare* with fig. 53) × 2000.

541 182.5.547 962

Bound Water and Phase Equilibria in Protein Systems: Egg Albumin and Muscle

By T MORAN (Low Temperature Research Station, Cambridge)

(*Communicated by Sir F Gowland Hopkins, O.M, P.R.S —Received June* 27, 1935)

This work is largely an extension of that already carried out on gelatin (Moran, 1926, 1932; Lloyd and Moran, 1934, and Hardy, 1926). These papers discussed (*a*) the crystal pattern and nature of the ice phase in frozen gels; (*b*) the amounts and nature of the water in equilibrium with unit mass of gelatin at different temperatures or activities of water

1—EGG ALBUMIN

The egg albumin was prepared by Hopkins's method, slightly modified as described by Adair and Robinson (1930). It was found that thorough washing with the solution prepared by adding 60 cc M sodium acetate solution and 40 cc of M acetate acid to 100 cc of saturated ammonium sulphate solution gave a sufficiently pure albumin since its water relations (as described later) were identical with those of an albumin recrystallized three times after this washing. The solution of albumin was dialysed under slight pressure at 0° C for several weeks until, in the final test, a 24-hour dialysate from approximately 60 cc of albumin solution, when concentrated to 1–2 cc, gave no reaction for sulphate. Several samples of concentrated albumin solution were prepared. The concentration (grams albumin per 100 grams of solution) varied from 20 to 33% with a Δ consistent at about 0 025°; the p_H as measured by the glass electrode was 4·8. The conductivity per 1% egg albumin never exceeded 2×10^{-6} mhos.

Freezing Equilibria

The water-binding capacity of gelatin at different activities of water can be measured by making use of the fact that when small discs of gel are slowly frozen ice forms wholly on the surface of the gel and can be easily removed, leaving a core of concentrated unfrozen gel (Moran, 1926) The water content of this gel is determined and the activity of the water in the gel at the reference temperature 0° C, calculated from the equation of

Lewis and Randall (1923). The activity-water curve varies slightly for different samples of gelatin, but fig 1 gives the results obtained with the sample of gelatin used in the present work The method is generally applicable to gels, and fig. 1 shows the results obtained with a 5% agar gel

Native Albumin

An attempt was made to apply the method indirectly to albumin sols. Albumin-gelatin gels were prepared with a known ratio of albumin to gelatin varying from 7 5 to 1 (the albumin and gelatin sols were mixed at a temperature not exceeding 35° C), and from the amount of water unfrozen per gram of dry protein (albumin and gelatin) it was possible, after allowing for the water held by the gelatin and assuming no interaction between the proteins, to calculate the amount of water associated with the albumin at different temperatures or activities of water. Within the experimental error the same curve was obtained for each gel and is shown in fig 1

An alternative method was devised to follow the water relations of egg albumin based on the same principle of freezing equilibria but avoiding the possibility of complications due to the presence of the gelatin. The albumin sol was placed in collodion bags (capacity approximately 20 cc), which were securely corked and buried in snow or chippings of ice. The bag and ice were then placed in a thermostat at 0° C, and the temperature lowered gradually (approximately 0·05° in two days) to − 0·5° C, at which temperature it was held for several days. It was found that as the temperature fell water passed out from the albumin sol through the membrane to swell the ice on the outside. The water content of the concentrated solution of albumin was determined at intervals until equilibrium was established at each temperature The temperature of the bath was then lowered to another temperature In this way values were obtained down to − 20° C, although at this temperature the albumin was like rock. The average of the results for two series of experiments are shown in fig 1 and in Table I.

Denatured Albumin

Several attempts were made to apply the two methods just described to heat coagulated egg albumin, but they were unsuccessful owing to the tendency for ice crystals to form within the gel or coagulum Albumin denatured by urea according to the method of Hopkins (1930) was, however, particularly easy to manipulate and gave conclusions reproducible within 0·5%. A convenient sample and method of preparation was to add 5 gm of urea dissolved in 4 cc of water to 4 cc of a 20 to 30% solution of albumin

contained in a weighing bottle, this sets to a firm rubber-like gel within an hour at ordinary temperatures The gel was left overnight and then cut into discs and these were dialysed in distilled water for 7 to 10 days at 0°, by which time there was no reaction for urea. The discs were then frozen and examined in the same manner as for gelatin gel The values obtained are given in Table I and fig 1. It will be observed that the water-binding

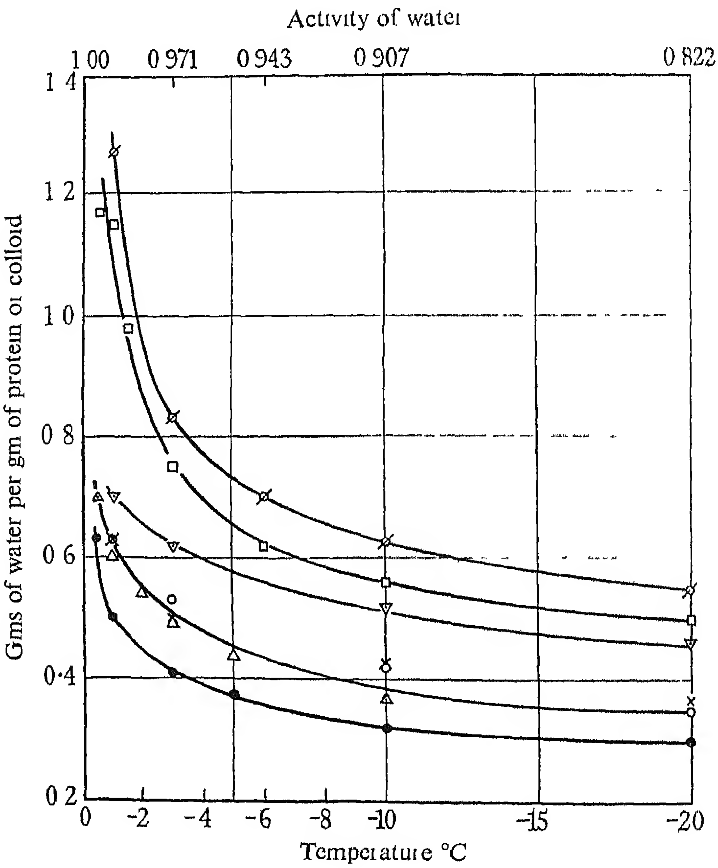


FIG 1—ø Agar agar, ⊡ gelatin, ▽ myogen, △ × ⊙ native albumin, ● denatured albumin, ⊙ gelatin-albumin gel discs, △ × collodion sac method.

capacity of denatured albumin at different activities of water is smaller than for native albumin.

It was observed previously (Moran, 1926) that the curve for gelatin is a reversible one. The same was found to be true of denatured egg albumin between − 20° and − 1° C. The curve for native egg albumin could not be tested for reversibility.

It is difficult to carry out experiments requiring actual manipulation of the material at temperatures much below − 20° C, but the relationship between the water content of the colloid and the activity of water (and therefore freezing temperature) can also be investigated by a pressure method, which, in principle, is the same as that of the freezing technique. The application of the method to gelatin gels has been described in detail (Lloyd and Moran, 1934) It consists in determining the water content of the colloid at equilibrium, the colloid being under an applied pressure P

TABLE I

Temp °C	Activity of water	Grams water per gram colloid						
		Gelatin	Agar	Native albumin (gel exp)	Native albumin sac I	Native albumin sac II	Urea albumin	Myogen
− 0 5	0 995	1·17	—	—	—	0 70	0 63	—
− 1	0 990	1 15	1 27	0 63	0·63	0·60	0 50	0·70
− 1·5	0 986	0 98	—	—	—	—	—	—
− 2	0·980	—	—	—	—	0 54	—	—
− 3	0 971	0 75	0 83	0 53	0 50	0 49	0·41	0 62
− 5	0·953	—	—	—	—	0 44	0·375	—
− 6	0 943	0 62	0 70	—	—	—	—	—
10	0 907	0 56	0 63	0 42	0 43	0 37	0·32	0 52
20	0 822	0 5	0 55	0 35	0·37	—	0 30	0·46
45	0 640	0 5	—	—	—	—	—	—

and separated by a semi-permeable membrane from water under atmospheric pressure The relationship between the activity of the water and the pressure P is given by the equation

$$\left(\frac{\partial \ln \alpha}{\partial P}\right)_T = \frac{V}{RT},$$

where V is the molal volume of the water in the system.

The same technique, precisely, has been used in the present work. The experiments with heat- and urea-denatured egg albumin were straightforward, but with native egg albumin it was necessary first to concentrate the solution by slight freezing (the sac method) until it was practically solid (concentration by weight approximately 50%) The pressures in each experiment were applied for 7 to 10 days at 0° C, equilibrium being apparent over the final three days The results are given in Table II, the concentration of the residual protein at the different pressures being expressed as grams of water per gram dry protein For comparison, the values obtained by the freezing technique at the higher activities are also shown.

Egg White

St John (1931), using a calorimetric technique, concluded that in the thick white of an egg there is a considerable amount of bound water, equivalent to 1·92 grams per gram of dry solid at temperatures below −12·5° C. Jones and Gortner (1931) arrived at a similar conclusion, using the dilatometer method and at − 20° C found the bound water to be approximately 1·33 grams per gram dry solid A J. M. Smith (1933), however, has recently reinvestigated the problem, using the calorimetric technique, and concluded that the unfrozen water at − 21° C in thick egg white is approximately 5% of the total, equivalent to 0 37 grams per gram dry solid

TABLE II

Pressure lb/sq in	Activity of water	Pressure experiments: Native albumin	Pressure experiments: Heat denatured albumin	Pressure experiments: Urea albumin	Freezing experiments: Native albumin	Freezing experiments: Urea albumin
500	0 976	0 53	—	0·48	0 51	0 43
1000	0·952	0 46	0·44	0·38	0 46	0·38
2000	0 904	0·38	0 33	0·34	0·38	0·32
13,000	0·513	0·25	0 25	0·24		
32,000	0 195	0 23	0·24	0·23		
38,000	0 146	0·22	—	—		

The main constituent of egg white is albumin, and in view of the foregoing on native albumin it would be expected that the bound water at − 20° C would be approximately 0 35 grams per gram solid. On the other hand, the eutectic of egg white is below − 20° C (approximately − 31° C, *cf.* later in this paper), so that there will be a small amount of concentrated salt solution present in addition. The amounts of water unfrozen in thick and thin egg white at different temperatures have been determined using the collodion sac method. The initial water contents were 88 0 and 88 45% respectively, with a Δ in each case of 0·42°; it was found that the water contents at different freezing temperatures were identical, indicating that the bound water is approximately the same in each sample. The actual figures are shown in Table III.

At −20° C, therefore, the bound water of egg white (thick and thin), does not exceed 0 41 grams per gram solid. A criticism of this method applied to systems containing salts is that there will be a loss of salts through the collodion membrane This, however, is small and probably has a negligible effect on the equilibrium. Thus, the original Δ for the egg white was

TABLE III

Temperature °C	Water content of concentrated white
− 0 75	82 6
− 1	76·8
− 3	51 4
− 10	35 2
− 20	29 2

0·42° C. At the completion of the experiment a weighed sample was crushed and dissolved in sufficient water to reproduce the original white. Δ for this solution was 0·35° C.

II—MUSCLE

Muscle contains at least three soluble proteins myogen, an albumin, and two globulins, myosin and another, recently isolated (Meyer, 1933) and differing from myosin in the fact that it is soluble in smaller concentrations of salt. The crystalloid content of the expressed juice of rabbit's muscle has been investigated by E. C. Smith (1934), who concludes that the dispersing power of the crystalloids in living muscle is equivalent to that of 0·18 M KCl From the solubility of myosin in solutions of KCl, Smith concludes that both living and dead muscle are virtually suspensions of solid myosin (probably particles of weak gel) in an aqueous medium containing salts, soluble protein, and other nitrogenous substances.

In a previous paper (Moran, 1930), the amount of water remaining unfrozen per gram of dry solids in muscle at different temperatures was determined by a dilatometric method and it was found that the amount of bound water was small; at − 20° C (corresponding to an activity of 0·822) it did not exceed 6% of the water in the muscle or the equivalent of 0·25 gm of water per gram of dry solid.

The dilatometer method has the limitation that it is difficult to allow for the changes in volume due to the de-solution of dissolved air or CO_2, when the bulk of the material is frozen. An attempt has been made to avoid this source of error by using a simple method based on the sac experiments with albumin.

Ox Muscle

This muscle freezes at − 1° C and accordingly small cubes of muscle (2 × 2 × 1 cm approximately) were embedded in a 15 to 20% gelatin gel made isotonic with the muscle by the introduction of sodium chloride.*

* In actual practice the amount of salt added was somewhat below the calculated amount, but the gel was first frozen to equilibrium at − 1° C and the ice that separated was removed The gel remaining was used

The cubes of gel and muscle were frozen slowly and stored at different temperatures Ice formed only on the outer surface of the layer of gelatin gel, leaving behind concentrated gel and partially dehydrated muscle When equilibrium was reached at a particular temperature, the water content of the muscle was measured Table IV shows the values obtained.

Amphibian Muscle

In these experiments freshly excised gastrocnemius muscles were embedded in a 15% gelatin gel in Ringer solution made up of NaCl 0 675%, $CaCl_2$ 0 020%, and KCl 0·015% at 30° C (Hill and Kupalov, 1930), rapidly cooled to 0°C for some minutes and then frozen at − 1° C. Muscles removed from these blocks were irritable after several hours so that at freezing the muscles were living. It has been shown that freezing at or below − 2° C immediately kills the muscle (Moran, 1929), but it was suggested to me by Sir William Hardy that the changes in structure consequent on death do not take place until the muscle is thawed. There appears to be no method of testing this theory; but it is possible that the results obtained with this muscle are characteristic of living muscle. They are given in Table IV

TABLE IV

Equilibrium temperature °C	Ox muscle		Frog's gastrocnemius muscle	
	Water content of muscle	Unfrozen water per gm solid	Water content	Unfrozen water per gm solid
− 0 42	76 4	3 20	79 9	3 98
− 1 0	76 4	3 20	—	—
− 2	—	—	48 8	0·95
− 3	50 6	1 00	42 3	0 73
− 5	42 3	0·73	36 8	0·58
− 10	35 1	0 54	32·3	0 48
− 20	29 4	0·41	31 3	0·46
− 40	30 0	0 43	31·0	0 45

It is shown later that the eutectic of the muscle crystalloids is approximately − 37 5° C If the mass of the eutectic solid phase can be neglected, the water " unfrozen " at − 40° C represents bound water, and at this activity (approximately 0 7) corresponds to 0 40 to 0·45 gm of water per gram of dry solid. This figure is roughly that found for pure myogen, fig 1, the bound water of this protein at different activities of water was determined

by the freezing method in the presence of gelatin as described for native egg albumin

There is no method of calculating exactly the bound water in muscle at higher activities because of the difficulty of allowing for the amount of water necessary to dissolve the salts in the muscle. The evidence is, however, that even in unfrozen frog's muscle (activity = 0 994) the amount of bound water is small and of the same order as that found at intermediate activities (Brooks, 1934). Hill and Kupalov (1930) point out that the osmotic pressure of living muscle can be accounted for by assuming the soluble constituents to be dissolved in *all* the water. from this vapour pressure technique they concluded that the bound water does not exceed about 0·15 gm per gram of solid In fatigued muscle, or muscle in rigor, the observed osmotic pressure of the muscle cannot, at present, be satisfactorily accounted for on the basis of the sum of the total soluble constituents. Brooks (1934), however, from an analysis of the vapour pressure isotherm of dead sartorius muscle calculated the bound water in unfrozen muscle to be 0 29 gm per gram solid.

At low activities it is possible to arrive at a measure of the bound water of muscle by the pressure method. One pressure was investigated very carefully, 13,300 lb per sq inch corresponding to an activity of 0 50 approximately The pressure was applied at a constant rate and the following are details of one rigorous experiment carried out at 0° C.

Mass of ox muscle used = 60 gm.
Water content of muscle = 76 25%
Pressure applied (*a*) to 2000 lb per sq inch in 24 hours and then maintained for 6 days, (*b*) to 13,300 lb in 24 hours and maintained for 14 days
Volume of expressed fluid collected * = 30 8 cc.
Δ of juice* = 0 98.
Water content of muscle remaining = 25 01%
Bound water of muscle = 0 33 gm per gram solid

An experiment carried out under similar conditions at a pressure of 2000 lb per sq inch corresponding to freezing at − 10° C gave a residue of ox muscle containing 0·51 gm of water per gram solid. This figure includes a certain amount of free water necessary to dissolve the salts present.

The evidence here with that of Hill and Brooks would appear to be satisfactory proof that the bound water in muscle, living or dead, is

* Corrected for water lost by collodion-treated canvas

relatively small and probably does not exceed about 0·4 gm per gram of dry solid, or about 10% of the total water in the muscle.

The Eutectic of Muscle

Heiss (1933), from the cooling curve of muscle frozen at different temperatures, concluded that the eutectic of muscle (the temperature at which all the free water is frozen) is − 62° to − 65° C. Brooks (1934) calculated that the free water is negligible at an activity of water of 0·5, corresponding to a temperature in the range − 40° to − 60° C. The eutectic temperature of salt solutions can be determined quite readily with an accuracy of about 1° C by following the changes in electrical resistance as the temperature is lowered of a column or layer of the salt solution (Moran, 1934). At the eutectic temperature the resistance increases rapidly. Similarly, on thawing, there is a sudden and rapid fall in resistance. From observations on (1) a concentrated ultra filtrate of muscle juice, (2) a concentrated solution of extracted muscle salts, it was concluded that the eutectic temperature of muscle is approximately − 37·5° C.

Discussion

This work shows that native or denatured egg albumin, muscle, myogen, and agar agar, like gelatin, bind a certain amount of water and the amount so held is governed by the activity of the water. This conclusion is almost certainly a general one applying to all colloids (*cf.* also Briggs, 1931).

In the presence of salts or other dissolved substances the water relations of a protein may be altered very considerably. This has been shown for gelatin (Moran, 1932) and for egg albumin and haemoglobin (Adair and Moran, 1934). Thus, in the presence of saturated sodium chloride solution at 0° or 20° C, corresponding to an activity of water of 0·81, the apparent hydration of egg albumin is approximately 0·13 as compared with 0·35, the value found in the present work. Incidentally, these experiments indicate that at high activities temperature has little effect on the bound water of a protein.

At low activities of water in the absence of salts, the water held by native or denatured egg albumin is approximately the same and equal to 0·26 gm per gram of protein. By analogy with gelatin (Moran, 1932), it is suggested that this is the chemically bound water of albumin; the figure is near to that found by Sorensen (1917) for the water of hydration of egg albumin crystals, namely, 0·22. At high activities denatured albumin binds less water than native albumin.

Mr. W. T. Astbury tells me that this is in agreement with recent observations of his on the X-ray photographs of native and denatured proteins In the completely denatured protein the peptide chains are aggregated into bundles or crystallites held together by backbone and side-chain linkages These linkages, particularly those between side chains, restrict the entry of water molecules into the structure of the protein.

It is difficult to arrive at any measure of the hydration of a protein from the hydration of its constituent amino acids, since several workers (Lewis, 1931; Margaria, 1931; Hoskins, Randall, and Schmidt, 1930, Frankel, 1930) have shown by freezing-point, or vapour pressure, determinations that many of these acids including glycine, aspartic acid,

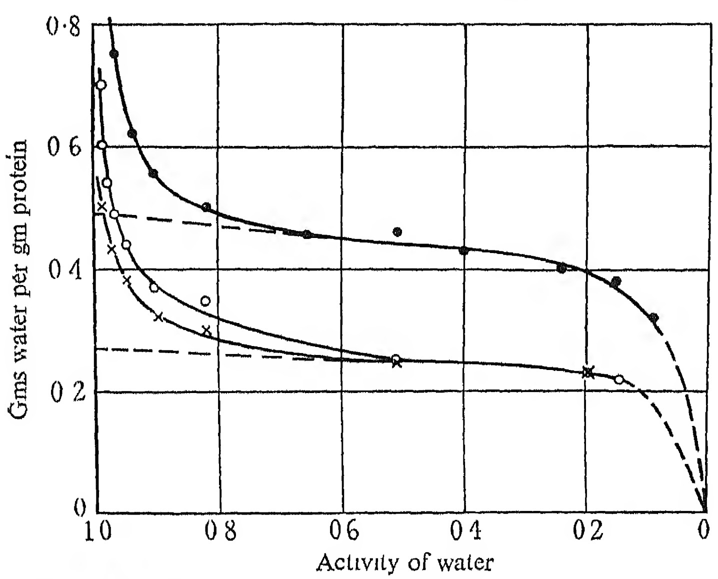


FIG. 2—● gelatin, ○ native egg albumin, × denatured egg albumin.

glutamic acid, arginine, and phenylalanine are, in effect, polymerized in solution; any hydration is therefore masked by this factor

Only three determinations were made of the percentage denaturation in native albumin submitted to pressure These showed that the denaturation increased with increase of pressure or decrease in activity of water The figures obtained were 2·8, 5 9, and 9·1% after one week at 0° C under pressures of 1000, 13,000, and 38,000 lb per sq inch respectively

The complete curve relating activity of water to the bound water of either gelatin or albumin shows a pronounced pseudo-plateau, fig 2. Data are not available to construct the vapour pressure isotherms of pure gelatin and albumin at 0° C, but the results of Katz (1917) and Barker (1933) at

higher temperatures (approximately 23° C) do not indicate a plateau in these curves On the other hand, Brooks (1934) found the vapour pressure isotherm at 0° of dead sartorius muscle to be reversible and with a definite plateau over the range of activity of water 0·5 to 0·1

Hardy (1926), in considering the plateau in the freezing curve, suggested that it might be explained on the increased frictional resistance in the gel which stops the passage of water to the ice face. The difficulty in accepting this explanation, at least down to activities of about 0·65, is, as Hardy pointed out, that the curve is a reversible one At low activities, however, where it is impossible to test for reversibility, viscosity may play a part in determining the equilibrium conditions.

A further possibility is that the gel framework exerts an opposing pressure which increases with increasing concentration of colloid and reaches a limiting value when the protein molecules with their extending side chains and "anchored" water molecules are all touching Either theory, extremely slow attainment of equilibrium or mechanical resistance, would still suggest that in the region of the plateau the water is very firmly held, and in view of the results with gelatin in the presence of salts (Moran, 1932), probably by covalencies On the other hand, if either factor plays a part, the simple thermodynamic treatment relating activity of water to applied pressure would have to be modified. It is proposed to determine accurately at 0° C the vapour pressure isotherms of the pure colloids used in these experiments

The bound water of native egg albumin and egg albumin denatured by heat and by urea has been determined at different activities of water from observations on the equilibrium in the frozen state and under an applied pressure. The chemically bound water of the two proteins is approximately the same (0·26 gm per gram of protein), but the water held more loosely at high activities is less in the denatured protein.

Measurements have been made of the water content of muscle in equilibrium with ice at different temperatures and activities of water

The eutectic temperature of muscle is approximately − 37·5° C The bound water of muscle is small and at intermediate and high activities of water is of the order of 0·40 gm per gram of dry solid.

References

Adair, G S, and Moran, T (1934) "Rep Food Invest Bd," p. 29.
Adair and Robinson (1930) 'Biochem J,' vol 24, p. 993

Barker, H A. (1933) 'J Gen Physiol,' vol **17**, p 21
Briggs, D R (1931) 'J Phys Chem,' vol **35**, p. 2914.
Brooks, J. (1934) 'Proc. Roy Soc,' B, vol **114**, p 258.
Brooks, J. (1934). 'J. Gen Physiol,' vol. **17**, p 783
Frankel (1930) 'Biochem Z,' vol **217**, p 378
Heiss (1933). 'Z ges Kälteindustr,' vol **40**, p 97
Hill, A V, and Kupalov (1930) 'Proc Roy. Soc,' B, vol **106**, p 445
Hopkins, Sir Frederick G. (1930). 'Nature,' vol. **126**, p 328
Hoskins, Randall, and Schmidt (1930). 'J Biol Chem,' vol **88**, p 215
Jones and Gortner (1931) "Colloid Symposium Monograph," vol **9**, p. 387
Katz (1917) 'Kolloidchem. Beih,' p 1.
Lewis, W. C M (1931). 'Chem. Rev,' vol **8**, p. 81.
Lewis and Randall (1923) "Thermodynamics," p. 255
Lloyd, D Jordan, and Moran, T. (1934) 'Proc. Roy. Soc. A, vol **147**, p. 382
Margaria (1931) 'J. Physiol,' vol **73**, p. 311.
Meyer, K. (1933) 'Biochem. Z.,' vol **266**, p 137.
Moran, T (1926). 'Proc. Roy Soc,' A, vol. **112**, p 30.
Moran, T. (1929). *Ibid*, B, vol. **105**, p 177.
Moran, T (1930) *Ibid*, B, vol **107**, p. 182
Moran, T (1932) *Ibid.*, A, vol. **135**, p. 411
Moran, T (1934). "Rep. Food Invest. Bd," p. 27
St John (1931) 'J. Amer. Chem Soc,' vol **53**, p 4014.
Smith, A J M (1933) "Rep Food Invest Bd.," p 113.
Smith, E C. (1934) 'Proc Roy Soc,' B, vol. **114**, p. 494
Sorensen, S P L (1917). 'C R Lab Carlsberg,' vol. **12**.

547 963 4 · 542 943

The Oxidation of Haemoglobin to Methaemoglobin by Oxygen

II—The Relation between the Rate of Oxidation and the Partial Pressure of Oxygen

By J Brooks (Food Investigation Board of the Department of Scientific and Industrial Research, and Low Temperature Research Station, Cambridge)

(*Communicated by D. Keilin, F.R S.—Received July* 19, 1935)

Neill and Hastings (1925) found that the rate of oxidation of haemoglobin by dissolved oxygen in aqueous solution was maximal at a small partial pressure of oxygen; a similar result was obtained (Brooks, 1929) for the oxidation of haemoglobin in pigmented muscle In both cases the rate was determined approximately A possible explanation of the effect was suggested to Neill and Hastings by the work of Conant (1923, 1924–25, 1926, 1928) who had shown that methaemoglobin and reduced haemoglobin formed an oxidation-reduction system of the ferri-ferro type. If only reduced haemoglobin reacts with oxygen to give methaemoglobin, increasing the oxygen pressure decreases the concentration of the other reactant (by the formation of oxyhaemoglobin), and a maximum rate of oxidation might be obtained.*

The rate of oxidation of haemoglobin, using ox-blood diluted in a phosphate buffer, was measured at a single oxygen pressure (Brooks, 1931) and found to be unimolecular with respect to the concentration of haemoglobin Similar measurements at 30° C and $p_{\rm H}$ 5·69 have now been made at other oxygen pressures, the concentration of oxy- and reduced haemoglobin in the system has also been determined. Fortunately, the establishment of equilibrium between oxygen and haemoglobin is very rapid in comparison with the rate of oxidation (Hartridge and Roughton, 1923, 1925).

* Haemoglobin is used to denote both the ferro-compounds, oxy- and reduced haemoglobin As methaemoglobin contains no more oxygen than reduced haemoglobin the formation of the ferri-compound must involve either a preliminary or simultaneous separation of one oxygen molecule per atom of iron from oxyhaemoglobin

MATERIAL AND METHODS

The same general method was used to determine the rate of oxidation and the oxygen-dissociation curve of haemoglobin. A mixture of oxygen and nitrogen was passed over a shallow layer of dilute haemoglobin solution shaking slowly to and fro, and portions of the solution were analysed at intervals by the gasometric methods developed by van Slyke Some of the details given below are more fully described in a previous paper (Brooks, 1931).

Blood—Fresh defibrinated ox-blood was laked by repeated freezing and thawing. The same stock of laked blood was used throughout as blood from different animals gave different values for the rate of oxidation The laked blood was kept at − 20° C and portions thawed when required.

Buffer Mixtures—The haemoglobin solutions were heavily buffered with K_2HPO_4 and KH_2PO_4; the total concentration of phosphate was always 0·6 M and the $[HPO_4^{=}]/[H_2PO_4^{-}]$ ratio 0·159/0·841 Interpolation of Cohn's (1927) data gives the p_H at 30° C as 5·69 (taking a temperature coefficient of − 0·03 p_H for an increase of 10° C).

The use of a heavily-buffered solution has two advantages: (*a*) there is no danger of bacterial contamination during the experiment, (*b*) the change in p_H (*cf.* Henderson, 1920), due to different concentrations of oxy- and reduced haemoglobin at different oxygen pressures, is negligible. The initial concentration of haemoglobin in the solution was roughly 3 gm per 100 cc, or 0 00045 M. It can be shown that a change from the completely reduced to the completely oxygenated form would change the p_H by less than 0 005.

Methods of Analysis—The manometric apparatus of van Slyke and Neill (1924) was used to measure the following concentrations:

(*a*) Haemoglobin—from the CO-combining capacity determined by the method of van Slyke and Hiller (1928).

(*b*) Total pigment—from the CO-combining capacity after reduction with sodium hydrosulphite determined by the method of van Slyke and Hiller (1929).

(*c*) Undenatured pigment—from the O_2-combining capacity after reduction with titanous tartrate by a slight modification of the method of Conant, Scott, and Douglas (1928).

(*d*) Oxyhaemoglobin—from the O_2-content determined by the method of van Slyke and Neill (1924).

In each case 2 cc of the solution were analysed The necessary corrections were determined by repeating the analyses with 2 0 cc of a solution of the

same phosphate composition as the haemoglobin solution Because of the high concentration of phosphate the more acid ferricyanide reagent of van Slyke and Hiller (1929) was used in the determination of (*a*), the reagents used in (*b*) were also slightly modified (Brooks, 1931)

Results are given in vols per cent, *i.e* , cc CO or O_2 (0° C, 760 mm Hg), combined with or liberated from the pigment contained in 100 cc solution The agreement between duplicate analyses of (*a*) or (*d*) was within 0·03 vols per cent, and for (*b*) or (*c*) within 0 10 vols per cent

The concentration of unchanged haemoglobin is given by (*a*), the methaemoglobin concentration by (*c*) minus (*a*), and the concentration of other products, *e g*., cathaemoglobin and haematin, by (*b*) minus (*c*). As the concentrations of total and undenatured pigment did not change during the oxidation, the formation of methaemoglobin is the only detectable reaction and the rate is given by the decrease in (*a*) with time

The fraction of haemoglobin in the form of oxyhaemoglobin is given by the ratio (*d*)/(*a*).

Apparatus—The all-glass apparatus used is shown in fig 1. The vessel A was immersed in a thermostat at 30° C (± 0·01° C), and contained a shallow layer of haemoglobin solution over which the gas mixture was passed. When the large-bore tap B was opened for the withdrawal of a sample by means of a pipette the gas-stream escaped through this tap instead of through the trap C, and so prevented any change in the composition of the gas mixture in A Before entering A the gas passed through the spiral D and the vessel E (which contained a solution of the same phosphate composition as the haemoglobin solution) The vessels A, D, and E were mounted on a horizontal steel rod which moved slowly to and fro and were connected to the rest of the apparatus by the bends of glass tubing F These bends were sufficiently non-rigid to accommodate the movement of A, D, and E

The gas mixtures were prepared from cylinders of either air and oxygen or air and nitrogen. Each gas was passed through cotton wool (G), alkaline $KMnO_4$ solution (H), and a half-saturated solution of NaCl* (I). The rate of flow of the two gases was measured by flow-meters K and L, and that of the mixture by flow-meter J Gas was continuously bubbled to waste from each of the three overflow-tubes M, N, and O; the composition of the gas mixture and its rate of flow was regulated by varying the depth of immersion of these tubes in the thermostat

The flow-meters were calibrated before assembly; a reading of 1 cm

* If water is used in I, moisture may be deposited in the capillaries of the flow-meters

(a purified paraffin was used) corresponded to a rate of about 0 4 litre per hour, and the readings of K and L remained constant to within 0 02 cm after setting the overflow tubes. The total rate of flow through both K and L was always about 7 litres per hour. The oxygen content of the mixture using air and nitrogen varied therefore by less than 0 02 vols per cent (0 15 mm Hg), and with air and oxygen by less than 0·1 vols per cent (0·75 mm Hg) The rate of flow of the mixture through A was about

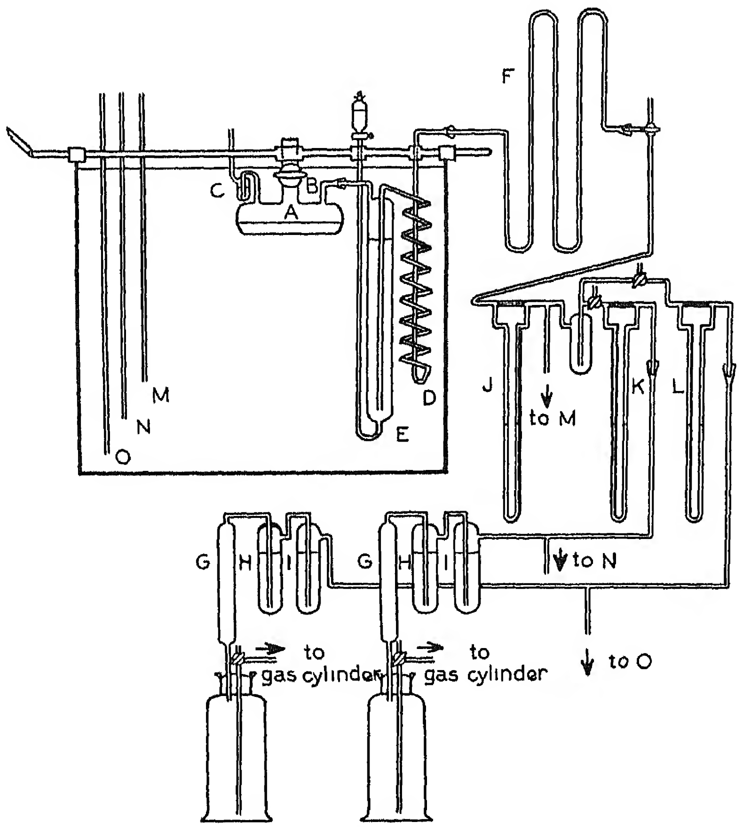


FIG 1.

6 litres per hour. Increasing the rate of shaking of A or the rate of flow of the gas mixture over the haemoglobin solution did not alter the rate of oxidation.

The cylinder nitrogen contained a small amount of oxygen (less than 1%), no attempt was made to remove it. The oxygen content of the gas from each cylinder used was determined by a modification of the method of van Slyke and Sendroy (1932), and the analyses are believed to be

accurate to within 0 03 vols per cent. The lower oxygen pressures (4 5 and 6 0 mm) were obtained by the use of cylinder nitrogen alone.

The Rate of Oxidation of Haemoglobin

In vessel A was placed the necessary amount of phosphate solution to give the desired concentration of KH_2PO_4 and K_2HPO_4 after the addition of a given volume of thawed blood The gas mixture was passed through the apparatus for 4–6 hours before adding the blood, and the first sample taken 1–2 hours later A volume sufficient for two determinations was withdrawn and placed in a vessel immersed in melting ice, oxygen was then bubbled through the solution for one minute There was no detectable change in the cooled solution during the time required for duplicate analyses

Table I gives the values for total and undenatured pigment at three oxygen pressures

Table I—Total Phosphate 0 6 M, p_H 5·69, 30° C

Oxygen pressure	Time	Total pigment	Undenatured pigment
mm	hours	vols per cent	vols per cent
6·0	0	5 74	5 59
	3·5	5·69	5·60
	8 6	5·76	5·63
93	0	5 75	5 69
	12	5 80	5 64
723	0	5·79	5·61
	4	5·80	5·69
	24	5·70	5 68

These results agree with those previously obtained (Brooks, 1931). There is no trend in the difference between the values in the last two columns, and the values for undenatured pigment are slightly lower than those for total pigment (this difference is referred to later).

Table II gives the decrease with time in the concentration of haemoglobin at different oxygen pressures (p). For convenience, concentrations are expressed as the logarithms of the percentage of unchanged haemoglobin, $\log_{10}(a - x)$, since two runs were carried out at most of the oxygen pressures and the initial concentration of haemoglobin varied from 5·0 to 3·0 vols per cent.* Times are given in hours from the

* There was a very slow but appreciable oxidation of haemoglobin in the frozen blood at $-20°$ C.

TABLE II—TOTAL PHOSPHATE 0 6 M, p_H 5 69, 30° C

Time Hours	$\log_{10}(a - x)$	$k \times 10^{-2}$
	p 723 mm	
0	2 000	
3 20	1 936	4 61
5 53	1 891	4·54
8 42	1 832	4 60
10 55	1 787	4 65
23 5	1 509	4 81
	p 435 mm	
0	2 000	
4 00	1 911	5 12
6·20	1 863	5 09
11·47	1·740	5 22
24 2	1·471	5 04
	p = 152 mm	
0	2·000	
2 80	1 924	6 25
4 35	1 880	6 35
6·10	1 832	6 34
11 15	1 689	6 43
23 6	1 309	6 74
	p - 122 mm	
0	2 000	
4 18	1 866	7 38
10 15	1·675	7 38
23 5	1 249	7 36
	p 92 9 mm	
0	2 000	
2 35	1 914	8 43
3 40	1 874	8 54
6 45	1 763	8 46
9·72	1 650	8 29
	p 63 1 mm	
0	2 000	
2 87	1 872	10·3
3·28	1 860	9 8
5 37	1 760	10 3
9 08	1 593	10 3

Time Hours	$\log_{10}(a - x)$	$k \times 10^{-2}$
	p - 33·3 mm	
0	2·000	
2 35	1 852	14 5
3 17	1 799	14·6
4 65	1 712	14 3
	p - 25·8 mm	
0	2 000	
1 39	1 893	17 7
3 02	1 768	17·7
4·60	1 647	17 7
6 42	1 503	17 8
	p = 16 0 mm	
0	2 000	
2 08	1 841	17 6
4 03	1 693	17 5
7 40	1 436	17·6
	p = 13 7 mm	
0	2 000	
1 33	1 911	15 4
2 96	1 791	16 3
4 72	1 675	15 9
	p = 6 0 mm	
0	2 000	
1 98	1 892	12 6
5 00	1 727	12 6
6 45	1·647	12 6
10 03	1 443	12 8
	p = 4 5 mm	
0	2 000	
1 68	1 928	9 87
5 60	1 760	9 87
9 16	1 610	9 81

withdrawal of the first sample for analysis Each concentration is the mean of duplicate determinations.

Values of the velocity constant k obtained from the equation

$$k = \frac{2\ 303}{t} \log_{10} \frac{a}{a - x} \qquad (1)$$

are given in the table The values agree with the previously observed unimolecular course of the reaction with respect to the concentration of haemoglobin The velocity constants at different oxygen pressures (determined graphically) are given in Table III and, for oxygen pressures less than 200 mm, in fig 2

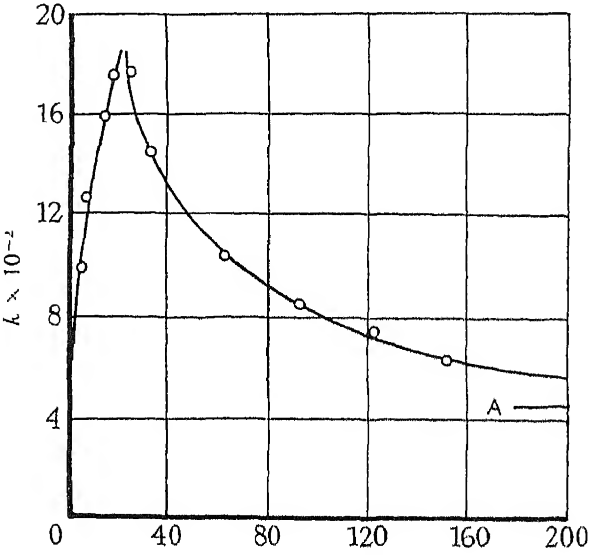


FIG 2—Velocity constants at different oxygen pressures A—value of k at 723 mm O_2.

TABLE III

p	4 5	6·0	13·7	16 0	25 8	33·3
$k \times 10^{-2}$	9 9	12 6	15 9	17·6	17 7	14·5
p	63 1	92 9	122	152	435	723
$k \times 10^{-2}$	10 3	8 45	7 40	6 36	5·17	4 61

There is a well-defined maximum rate of oxidation at approximately 20 mm oxygen. Increase of the oxygen pressure above roughly 200 mm has relatively little effect on the rate of oxidation.*

* The following control suggested to me by Dr Roughton shows that the oxidation is not a surface reaction proceeding at the interface between the solution and the gas phase At p = 723 mm some of the solution was withdrawn from the reaction vessel,

THE OXYGEN-DISSOCIATION CURVE OF HAEMOGLOBIN

The fraction of haemoglobin in the form of oxyhaemoglobin, denoted by $(1 - \alpha)$, at a given oxygen pressure was obtained from the following measurements:

(1) The concentration of haemoglobin (van Slyke and Hiller, 1928);
(2) The concentration of total oxygen, dissolved and combined (van Slyke and Neill, 1924),
(3) the concentration of dissolved oxygen;

and is given by $\frac{(2) - (3)}{(1)}$. Values of (3) were determined by separate experiments in which a 0·6 M total phosphate buffer, p_H 5·69, was equilibrated with several pressures of oxygen in the apparatus shown in fig. 1, and the dissolved oxygen measured by the same method used for total oxygen in (2). It was assumed that the solubility of oxygen in the phosphate buffer was the same as in the haemoglobin solution; this was supported by the values obtained for dissolved nitrogen (which were also determined in (2)); these were found to be the same in the two solutions The solubility of oxygen (and nitrogen) in the phosphate buffer obeyed the Henry-Dalton law within the limits of experimental error (Table IV).

TABLE IV—TOTAL PHOSPHATE 0·6 M, p_H 5·69, 30° C, NO HAEMOGLOBIN

Oxygen pressure (p)	Dissolved oxygen (c)	c/p
mm	vols per cent	
723	1 89	0 00262
152	0 41	0 00270
123	0 33	0 00268
58	0 15	0 00259

The haemoglobin solution was equilibrated with the gas mixture as described in the previous section A sample was withdrawn in a special pipette, and 2 cc were introduced into the gas-chamber of the van Slyke apparatus without contact with air; the total oxygen content was then determined (2). Immediately after the first sampling a second portion was withdrawn and placed in the cooled vessel previously described,

and a second vessel immersed in the thermostat was completely filled with this solution The amount of oxidation over two hours was the same in the reaction vessel as in the second vessel where no gas phase was present The change in concentration of dissolved oxygen over a short period would be negligible in the isolated solution.

oxygen was bubbled through the solution, and the CO-combining capacity (1) measured after the conclusion of analysis (2). The rate of oxidation at low pressures of oxygen is sufficiently rapid to introduce a serious error into $(1 - \alpha)$ if some method of "simultaneous" sampling is not used. A second value of $(1 - \alpha)$ was obtained from two further analyses, the agreement between the two independent values was within 0 01 (*i.e.*, within 1% saturation) The mean values* are given in Table V and, for oxygen pressures up to 58 mm, in fig. 3.

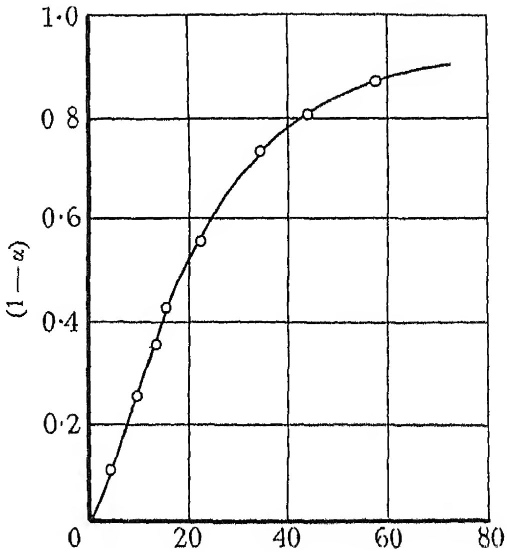


FIG 3—Oxygen dissociation curve of haemoglobin.

TABLE V—TOTAL PHOSPHATE 0 6 M, p_H 5·69, 30° C

p	4 5	10 0	13 7	16 0	22 5
$(1 - \alpha)$	0 109	0 258	0·360	0 430	0 558
p	34 6	44·0	58 0	123	152
$(1 - \alpha)$	0 733	0 808	0·874	0·938	0 952

* For a given oxygen pressure the percentage saturation is less than that usually found, due to the unusual salt concentration At p = 152 mm the percentage saturation is 95. This probably accounts for the discrepancy between the values for total and undenatured pigment. After reduction with titanous tartrate in the estimation of the latter, the equilibration of the only slightly diluted solution with air does not completely saturate the reduced haemoglobin with oxygen, and thus leads to a rather low result.

The points lie on a curve of slightly sigmoid shape (*cf* Forbes and Roughton, 1931), and for the range $p = 9 - 30$ mm fit Hill's (1910) empirical equation .

$$\frac{\alpha \quad p^n}{1 - \alpha} = K',$$

when $n = 1 \cdot 6$ and $K' = 114 \ 5$.

The Relation Between the Oxygen Pressure and the Rate of Oxidation

There are three possible paths for the reaction (*cf* Conant and Fieser, 1924–25) :

(1) The spontaneous decomposition of oxyhaemoglobin into methaemoglobin, O_2 and H_2O (or H_2O_2).
(2) The oxidation of reduced haemoglobin by oxyhaemoglobin.
(3) The oxidation of reduced haemoglobin by oxygen.

In (1) the rate would increase with increasing oxygen pressure and in (2) the reaction would be bimolecular with respect to the concentration of haemoglobin The pseudo-unimolecular course of the reaction and the maximum rate of oxidation suggest qualitatively a reaction between oxygen and reduced haemoglobin. Quantitatively there is not a simple relation between the rate, the concentration of reduced haemoglobin, and the oxygen pressure The following equation, however, fitted the experimental results :

$$\frac{dx}{dt} = k' \,.\, \alpha \quad (a - x) \,.\, \frac{bp}{1 + bp}, \tag{2}$$

where

k' = a constant = $1 \cdot 95$

α = the fraction of haemoglobin in the form of reduced haemoglobin

$(a - x)$ = the percentage of unoxidized haemoglobin at time t

b = a constant = $0 \cdot 0118$,

i.e , the rate of oxidation is proportional to the concentration of reduced haemoglobin and to a function of the oxygen pressure. Integration of (2) at a constant oxygen pressure gives (3)

$$\frac{k' \,.\, \alpha \,.\, bp}{1 + bp} = \frac{2 \cdot 303}{t} \log_{10} \frac{a}{a - x}, \tag{3}$$

and hence from (1)

$$k' \cdot \alpha \frac{bp}{1 + bp} = k \tag{4}$$

The agreement between observed and calculated values up to $p = 93$ mm is shown in Table VI. The first three columns of the table give the experimental values of p, k, and α, and the fourth column the values of k' calculated from (4), putting $b = 0 \cdot 0118$, k' should be independent of the oxygen pressure

TABLE VI

p	$k \times 10^{3}$	α	k'
4 5	9 9	0 89	2 21
6 0	12 6	0 85	2 24
13 7	15 9	0·64	1·79
16 0	17·6	0 57	1 94
25 8	17 7	0 39	1·94
33·3	14 5	0 29	1·77
63·1	10·3	0 12	2·01
92 9	8·45	0·08	2·02

At higher oxygen pressures the experimental error in the small value of α has a large effect on the calculated value of k'. It is preferable, therefore, to compare the calculated value of α (obtained from (4) putting $k' = 1 \cdot 95$ and $b = 0 \cdot 0118$) with the observed value This has been done in Table VII. A value of α determined at p 723 mm is included in the table *

TABLE VII

p	α observed	α calculated
63·1	0·12	0·124
92 9	0 08	0·083
122	0·06	0·064
152	0 05	0 051
723	0 03	0 026

The oxygen pressure p_m at which the rate of oxidation is maximal can be calculated when α is expressed in terms of p. From Hill's equation $\alpha = K'/\lambda\ (K' + p^n)$ Substituting in (4), differentiating k with respect to p and equating to zero gives (5)

$$K' - p_m{}^n (n - 1) - nbp_m{}^{n+1} = 0. \tag{5}$$

* This value of α is rather near the experimental error (0·01). As a check on the presence of systematic errors $(1 - \alpha)$ was determined at $p = 723$ mm in a phosphate buffer, p_H 5 69, concentration of total phosphate 0 2 M. At this phosphate concentration $(1 - \alpha) = 1$ 00 would be expected; the value found was 1 00.

From the known values of the constants in (5) $p_m = 19 \cdot 7$ mm. The observed value lies between 16·0 and 25·8 mm, and graphically (*cf* fig 2) is found to be approximately 20 mm

The agreement between observed and calculated values is satisfactory when the experimental errors over a wide range of oxygen pressure are considered. Up to $p = 63$ mm the approximately equivalent expression

$$\frac{dx}{dt} = k'' \, a \, . \, (a - x) \, . \, p^{0 \cdot 8}, \tag{6}$$

agrees with the results, but for higher pressures (2) is definitely superior. It should be mentioned that the use of an integral power of the oxygen pressure in (6) does not give even a qualitative agreement with the experimental results, *e.g.*, with the second power there would be no maximum rate of oxidation.

The Reactants in the Oxidation

The nature of the equilibrium between haemoglobin and oxygen is still uncertain, but the demonstration by Adair (1925) and Svedberg (1926, 1927), that the haemoglobin molecule contains four atoms of iron, probably leaves no alternative to the intermediate compound theory of Adair. In a haemoglobin solution, therefore, it is probable that the following compounds are present. Hb_4, Hb_4O_2, Hb_4O_4, Hb_4O_6 and Hb_4O_8. It is also possible that during the oxidation to methaemoglobin, compounds exist in which one or more of the four ferro-radicals have been oxidized to the ferri-state

The analytical results give the total number of ferro-radicals oxidized and the total number combined with oxygen, but do not differentiate between the molecular species which may be present. On this basis the rate of oxidation is proportional to the total number of ferro-radicals present which are not combined with oxygen $\propto (a - x)$, and to a function of the oxygen pressure $\dfrac{p}{1 + bp}$

In such a system there is a wide choice of possible reactants for the rate-determining step, but several of them may be excluded The pseudo-unimolecular course of the reaction indicates that (*a*) if the oxidation consists of a series of consecutive reactions, one of them is slow in comparison with the others; (*b*) if oxygen reacts simultaneously with more than one of the intermediates, Hb_4, Hb_4O_2, etc, the ferro-radicals concerned react at the same rate.

Equation (2) is not necessarily inconsistent with a simpler equation

$$\frac{dx}{dt} = k_2 \text{ [a particular intermediate] } p, \tag{7}$$

since the concentration of the intermediate may be written

$$\text{[an intermediate]} = \alpha\,(a - x)\,.\,f(p),$$

where $f(p)$ is an unknown function of the oxygen pressure which might possibly have the form $1/(1 + bp)$. This possibility of a simple reaction between oxygen and one of the intermediates is considered below

The concentration of the intermediates is given by the equations developed by Adair, but as the four equilibrium constants concerned cannot be evaluated independently it is possible to fit several sets of constants to a given oxygen-dissociation curve Using the notation of Ferry and Green (1929)

$$(1 - \alpha) = \frac{K_1p + 2K_1K_2p^2 + 3K_1K_2K_3p^3 + 4K_1K_2K_3K_4p^4}{4\,(1 + K_1p + K_1K_2p^2 + K_1K_2K_3p^3 + K_1K_2K_3K_4p^4)}. \tag{8}$$

Table VIII gives the observed and calculated values of $(1 - \alpha)$, using the following equilibrium constants* .

$$K_1 = 0{\cdot}1 \qquad K_3 = 0{\cdot}03$$
$$K_2 = 0{\cdot}05 \qquad K_4 = 0{\cdot}0667$$

TABLE VIII

p	$(1 - \alpha)$ observed	$(1 - \alpha)$ calculated
4·5	0·109	0·113
10·0	0·258	0·259
13·7	0·360	0·359
16·0	0·430	0·419
22·5	0·558	0·564
34·6	0·733	0·742
44·0	0·808	0·811
58·2	0·874	0·873
123	0·938	0·956

The fraction of haemoglobin in the form of the various intermediates can be calculated,† and these are given in Table IX.

* When p is very small (8) reduces to $(1 - \alpha) = K_1p/4(1 + K_1p)$. Values of $(1 - \alpha)$ between $p = 0$ and $p = 1$ were interpolated from fig 3 and fitted the simplified equation when $K_1 = 0{\cdot}1$ The other constants were determined by trial and error.

† $\alpha_{Hb_4} = 1/(1 + K_1p + K_1K_2p^2 + K_1K_2K_3p^3 + K_1K_2K_3K_4p^4) = 1/d$

$\alpha_{Hb_4O_2} = K_1p/d$

$\alpha_{Hb_4O_4} = K_1K_2p^2/d$

$\alpha_{Hb_4O_6} = K_1K_2K_3p^3/d$

$\alpha_{Hb_4O_8} = K_1K_2K_3K_4p^4/d$

It follows from equations (1) and (7) that

$$k_2\,[\text{an intermediate}]\,.\,p = k\,(a - x), \tag{9}$$

and from (9)

$$\alpha_i = \frac{k}{k'_2 p}, \tag{10}$$

where α_i is the fraction of haemoglobin in the form of the intermediate and k'_2 is a constant. Now k/p decreases with increasing oxygen pressure, *cf* Table X, third column Hence if the rate-determining step is the interaction of oxygen with one of the intermediates Hb_4, Hb_4O_2, etc, this intermediate must be Hb_4, since only for Hb_4 does α_i ($= k/k'_2p$) decrease with increasing oxygen pressure (Table IX) Hence from (10) $k/p\alpha_{Hb_4}$

TABLE IX

p	α_{Hb_4}	$\alpha_{Hb_4O_2}$	$\alpha_{Hb_4O_4}$	$\alpha_{Hb_4O_6}$	$\alpha_{Hb_4O_8}$
4·5	0·637	0 287	0·065	0 009	0 003
6 0	0·548	0 329	0 099	0·018	0 007
13 7	0 247	0 339	0 232	0 095	0 087
16·0	0 194	0·311	0·249	0 119	0 127
25 8	0·072	0 185	0·239	0 185	0 319
33 3	0·036	0·120	0 200	0 200	0 444
63 1	0 0045	0 028	0 089	0 169	0 710

should be a constant The last column of Table X shows that this is far from being the case, and it is concluded that the rate-determining step is not the reaction of oxygen with any one of the intermediates as given in (7). This conclusion is not affected by the use of other values of K_1, K_2, K_3, and K_4, since α_{Hb_4} is determined mainly by K_1, which can be evaluated independently with a sufficient degree of accuracy.

TABLE X

p	$k \times 10^{-2}$	k/p	$k/p\alpha_{Hb_4}$
4·5	9 9	2 20 × 10^{-2}	0 035
6 0	12 6	2 10	0 038
13 7	15 9	1 16	0 047
16 0	17 6	1·10	0 057
25·8	17 7	0 69	0 095
33 3	14 5	0 44	0 121
63·1	10 3	0 16	0·362

Similarly it can be shown that the substitution of p^2, p^3 or p^4 for p in (7) is not valid

The possibility of simultaneous reaction of oxygen with the ferro-radicals not combined with oxygen of more than one (but not of all) of the

intermediates cannot be investigated satisfactorily, since the concentrations of the intermediates, other than Hb_4, are uncertain The two following equations hold fairly well below $p = 63$ mm

$$\frac{dx}{dt} = k_3(4[Hb_4] + 2[Hb_4O_4])\ p$$

$$\frac{dx}{dt} = k_4(4[Hb_4] + 3[Hb_4O_2] + [Hb_4O_6])\ p,$$

but this limited agreement is probably fortuitous, since these two equations are not obeyed when another set of K_1, K_2, etc , values (which also fit the oxygen-dissociation curve) are used to calculate the concentration of the intermediates

These calculations, though incomplete, do not support the view that equation (2) can be explained by the reaction of oxygen with particular ferro-radicals, and it seems preferable to assume that all the unoxygenated ferro-radicals react at the same rate.* The term $p/(1 + bp)$ in the equation admits of at least two explanations; the more probable one is discussed in the next section, the other is the presence of an oxidative catalyst RO_2 in the system whose concentration is given by the equation $R + O_2 \rightleftharpoons RO_2$ (*cf.* Neill and Hastings, 1925). Hence

$$[RO_2] = \frac{p}{K + p} \cdot ([R] + [RO_2]) = \frac{p}{K + p} \cdot \text{constant}$$

If the rate of oxidation is proportional to the concentration of unoxygenated ferro-radicals and the catalyst RO_2, an equation of the same form as (2) would be obtained

* The term $bp/(1 + bp)$ has the same form as the expression for the fraction of oxygen molecules combined with one of the four types of ferro-radicals which is obtained when the combination of haemoglobin and oxygen is treated as a Langmuir adsorption on equal numbers of four different active spaces (*cf.* Hartridge and Roughton, 1925) On this basis

$$(1 - \alpha) = \tfrac{1}{4}\left(\frac{b_1 p}{1 + b_1 p} + \frac{b_2 p}{1 + b_2 p} + \frac{b_3 p}{1 + b_3 p} + \frac{b_4 p}{1 + b_4 p}\right),$$

this equation is the same as (8) when

$$K_1 = \Sigma\, b_1,\ K_2 = \Sigma\, b_1 b_2,\ K_3 = \Sigma\, b_1 b_2 b_3,\ K_4 = b_1 b_2 b_3 b_4.$$

This suggested that the oxidation was a surface reaction in which the rate-determining step was the reaction of an oxygen molecule attached to a particular ferro-radical with an adjacent ferro-radical not combined with oxygen. Quantitatively however, the $bp/(1 + bp)$ term in equation (2) could not be identified with any one of the four terms in the Langmuir equation, there are also objections to the particular surface mechanism which would obey equation (2) qualitatively

Discussion

It is known that oxygen is an inhibitor of many reactions, both thermal and photochemical The halogenation of many organic compounds is retarded by oxygen and the retardation by oxygen of the oxidation of oxalates by mercuric chloride has been studied by many investigators In the oxidation by dissolved oxygen of irradiated quinine in aqueous solution it was found (Weigert, 1912) that the rate of absorption of oxygen was thirty times greater from a gas-phase containing 0 6% oxygen than from pure oxygen. Solutions of fluorescein and erythrosin behaved in a similar way. In the latter reactions oxygen is both a reactant and an inhibitor This retardation by oxygen is usually attributed to either (*a*) the deactivation of activated molecules on collision with oxygen or (*b*), in chain reactions, to the breaking of chains by oxygen

Although the oxidation of haemoglobin is not light-sensitive to wave-lengths greater than 3100 A (Hasselbalch, 1909), there is ample evidence that the effect of inhibitors is the same in both thermal and photochemical reactions. Thus, Backstrom (1927, 1929) found that the inhibiting effect of alcohols on the rate of oxidation of sodium sulphite in aqueous solution was given by the equation

$$\frac{dx}{dt} = \frac{k_1}{k_2c + k_3},$$

where c is the concentration of the alcohol and k_2 and k_3 are constants common to both the thermal and photochemical oxidation The effect of inhibitors in other reactions has been expressed by similar equations, *e g*, in the thermal oxidation of oxalates by mercuric chloride in the presence of ferrous salts and oxygen (Roseveare and Olson, 1929)—

$$\frac{dx}{dt} = \frac{1}{k'_2\,(\mathrm{O}) + k'_3} + \frac{k'_4\,(\mathrm{O})^x}{k_5\,(\mathrm{O}) + k_6},$$

where (O) is the concentration of dissolved oxygen. The presence of two terms in this equation indicates that two reactions are proceeding simultaneously, and the effect of oxygen is threefold. (*a*) the inhibiting effect on the oxidation of oxalate by mercuric chloride shown in the denominator of the first term; (*b*) the oxidation of ferrous to ferric oxalate by oxygen shown in the numerator of the second term; and (*c*) the inhibiting effect on this second reaction shown in the denominator of the second term

The similarity between these equations and equation (2) suggests that in the oxidation of haemoglobin oxygen is both a reactant and an in-

hibitor, as in the oxidation of quinine. It has also a further effect an increase in the oxygen pressure decreases the concentration of the other reactant, unoxygenated ferro-radicals On this basis the rate of oxidation is proportional to the concentration of these radicals and the first power of the oxygen pressure, given by $\alpha(a - x)p$, and to the inhibiting effect of oxygen given by $1/(1 + bp)$

Further work would be necessary to determine whether the oxidation of haemoglobin is a chain reaction in which the chains are broken by oxygen molecules, or whether the postulated inhibition is due to the collision of oxygen molecules with an activated molecule or complex In either case, oxygen is not a very strong inhibitor,* and the small value of b when compared with unity in the term $(1 + bp)$ indicates that there is also present in the system a more efficient inhibiting mechanism which is not influenced by the oxygen pressure, *e.g.*, a constant concentration of "accidental" inhibitor or, in a chain reaction, breaking of the chains on collision with the walls of the vessel

Summary

The rate of oxidation of haemoglobin to methaemoglobin in a phosphate buffer, p_H 5 69, was measured at 30° C and several constant partial pressures of oxygen.

At each oxygen pressure the rate was unimolecular with respect to the concentration of haemoglobin. The formation of methaemoglobin was the only detectable reaction.

The fraction of haemoglobin in the form of oxyhaemoglobin at different oxygen pressures was determined under the same conditions of salt concentration, p_H, and temperature

The rate of oxidation at different oxygen pressures was proportional to the concentration of reduced haemoglobin (*i e*, the concentration of unoxygenated ferro-radicals) and to a function $\left(\frac{p}{1 + bp}\right)$ of the oxygen pressure

No evidence was obtained that the rate-determining step was the selective reaction of oxygen with one or more of the intermediate compounds of oxygen and haemoglobin. It was assumed that oxygen reacts at the same rate with all the unoxygenated ferro-radicals in the system

It was suggested that the effect of oxygen was threefold: (*a*) the determination of the concentration of the other reactant; (*b*) the oxidation of

* It will be seen from equation (2) that at very small oxygen pressures the rate of oxidation is proportional only to the concentration of unoxygenated ferro-radicals and the first power of the oxygen pressure

unoxygenated ferro-radicals, the rate being proportional to the first power of the oxygen pressure; and (*c*) the inhibition of the oxidation The results indicated that another inhibiting mechanism was present in the system

REFERENCES

Adair, G (1925) 'Proc Roy Soc,' A, vol **108**, p 627, vol **109**, p 292.

Alyea, H. N, and Backstrom, H. L J (1929). 'J Amer Chem Soc,' vol **51**, p. 90

Backstrom, H L J (1927) 'J Amer Chem Soc,' vol **49**, p 1460

Brooks, J (1929). 'Biochem J,' vol **23**, p 1391.

— — (1931) 'Proc Roy Soc,' B, vol **109**, p 35

Cohn, E J. (1927) 'J Amer. Chem Soc,' vol. **49**, p 173

Conant, J. B (1923) 'J Biol Chem.,' vol **57**, p 401

Conant, J B, and Fieser, L F (1924–25) 'J Biol Chem,' vol **62**, p 595

Conant, J B, and Scott, N D (1926) 'J Biol Chem,' vol **69**, p 575

—— (1928). 'J Biol. Chem,' vol. **76**, p. 207

Conant, J. B., Scott, N D., and Douglas, W F (1928) 'J Biol Chem,' vol **76**, p. 223

Ferry, R M, and Green, A. A (1929) 'J Biol Chem.,' vol **81**, p 175

Forbes, W H, and Roughton, F J W (1931) 'J Physiol,' vol **71**, p 229

Hartridge, H, and Roughton, F J W (1923) 'Proc Roy Soc,' A, vol **104**, p 395

— (1925) 'Proc Roy Soc,' A, vol **107**, p 654

Hasselbalch, K A (1909) 'Biochem Z,' vol **19**, p 435

Henderson, L. J (1920) 'J Biol Chem,' vol **41**, p 401

Hill, A V (1910). 'J. Physiol,' vol **40**, p. iv

Neill, J. M., and Hastings, A. B (1925) 'J Biol Chem,' vol **63**, p 479

Roseveare, W. E., and Olson, A R (1929). 'J Amer. Chem Soc,' vol **51**, p 1716

van Slyke, D D, and Neill, J M (1924). 'J Biol. Chem,' vol. **61**, p 523.

van Slyke, D D, and Hiller, A. (1928) 'J Biol Chem,' vol **78**, p 807

— (1929) 'J. Biol Chem.,' vol **84**, p 205

van Slyke, D D, and Sendroy, J, jun (1932) 'J Biol. Chem,' vol **95**, p 509.

Svedberg, T, and Fåhraeus, R. (1926) 'J Amer Chem. Soc,' vol. **48**, p 430.

Svedberg, T., and Nichols, J. B (1927) 'J Amer Chem Soc.,' vol **49**, p 2920.

Weigert, F. (1912) 'Festschrift, W Nernst,' Wilhelm Knapp, Halle, p 464

INDEX TO VOL. CXVIII (B)

END OF THE ONE HUNDRED AND EIGHTEENTH VOLUME (SERIES B).